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Prevalence of toxigenic mycobiota and mycotoxins in date palm fruits and investigation on *Bacillus cereus* 342-2 as biocontrol agent

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ABSTRACT

Date palm fruit is consumed as a highly nutritious food item particularly in the Middle East. This work was designed to investigate the occurrence of filamentous moulds, their toxins in date palm fruit samples, and explore the biocontrol potential of *Bacillus cereus* 342-2 against few date palms associated fungal species. In total, 36 date palm fruit samples from different sources and varieties were collected from the markets in Qatar. *Aspergillus* and *Penicillium* spp. were found to contaminate most of the samples, except Akhlas samples, which were free of any fungal contamination. In total, ochratoxin A (OTA) was found in 6 (16.67%) date samples, all within the permissible limit, except one (2.7%), where the level was above the maximum permissible concentrations of 2 µg/kg. The volatile organic compounds (VOCs) of *B. cereus* were very effective against the growth of *A. niger* and *A. carbonarius* with an inhibition rate of 100%. Whereas their effects against *A. flavus* was weak as mycelia grew; however, no sporulation occurred until day 7 of co-incubation. Gas chromatography mass spectrophotometry analysis of *B. cereus* volatiles showed aldehyde as a major component with a peak area of 10.6%, and Undecane with a peak area of 2.02%. The inhibitory effect of VOCs was either from aldehyde alone, or a synergetic effect of the two molecules.

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Highlights

- About 92% of the date palm fruit samples were contaminated with fungi
- AFs and OTA were detected in 5.56% and 16.67% of the tested samples
- Only 1 (2.78%) of the sample had mycotoxin level above the EU permissible limits
- Molecular identification of toxigenic fungi is an easy and reliable approach
- *B. cereus* 342-2 volatiles completely inhibited *A. niger* and *A. carbonarius*
- Bacterial volatiles were mix of hydrocarbons including alcohols and aldehydes

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This article has been corrected with minor changes. These changes do not impact the academic content of the article.

1. Introduction

Date fruit is an integral diet of the majority of the Arabian Gulf people. Qatar has approximately 1.5 million date palm trees which fulfil above 80% of the country's requirements (Muhammed et al., 2015). Freshly produced dates are either consumed directly or stored without preservation for off-season consumption. Unlike other agricultural products, date fruit is more prone to fungal infection especially due to the hot and humid environment during pre-harvest maturation stages. The contamination of date with fungal communities not only affects the physical characteristics, but also results in the deposition of mycotoxins.

Mycotoxins are a group of more than 300 fungal metabolites produced by toxigenic fungal communities belonging to different genera of filamentous mould (Galván et al., 2021). Fungal contamination of date palm fruit can also be associated with managemental practices such as long time storage without preservatives, open-air sun drying and stuffing with nuts (almond, peanuts), which are more prone to fungal infections. Shenasi et al. (2002) studied 25 varieties of date fruits at fresh and simulated storage stages for the presence of fungi and mycotoxins. At the early stage of maturation (called as Kimri) microbial count was lower compared to that of the second stage (Rutab), and was least at final stage (Tamr). Despite that 40% of the samples were contaminated with *A. flavus*, only 12% showed aflatoxins (AFs) contents.

Dietary intake of mycotoxins in human and animals is a major route of exposure and may pose a serious health risk as they may cause carcinogenic, mutagenic, teratogenic, atherogenic, and oestrogenic effects (Ozer et al., 2012). Moreover, it happens where one fungus produces more than one mycotoxin that might produce additive or synergistic toxicities to the exposed communities (Patriarca & Fernández Pinto, 2017). In Saudi Arabia, Gherbawy et al. (2012) reported aflatoxigenic *A. flavus* (7 out of 18 isolates) and ochratoxigenic *A. niger* (9 out of 36 isolates) associated with date palm fruits. Likewise, Shenasi et al. (2002) in Egypt reported AFB1 in 12% of the date palm samples at maximum levels of 417 µg/kg. In Yemen, Alghalibi and Shater (2004) found *A. niger* and *A. flavus* in date palm fruit along with 20% of the samples contaminated with AFB1 at 110–180 µg/kg. In a more extensive survey, Azaiez et al. (2015) analyzed 67 date palm fruit samples collected from Tunisia (40 samples) and Spain (27), and found 87% of the samples contaminated with at least one of 16 tested mycotoxins. OTA (average concentration of 1.26 µg/kg) and AFs were detected in 38% and 46%, respectively (Azaiez et al., 2015). In Pakistan, Luttfullah and Hussain (2011) found AFs in 2 out of 20 date palm fruit samples at an average concentration of 2.5 µg/kg. In the markets of Brazil, 1.5% of date palm fruits were contaminated with *A. niger*, while none of the samples were found positive for OTA (Iamanaka et al., 2005). Considering the toxicities of mycotoxins, many developing countries also have set strict regulations to control mycotoxins in foods. European Union (EU) has set regulation (Commission Regulation (EC), 2006) for aflatoxin B1 (AFB1) and total AFs in processed dried fruit as 2 and 4 µg/kg, respectively.

In the last few decades, to ensure food safety, there has been a huge focus on developing efficient technologies to limit the fungal attack and synthesis of toxic metabolites in cereals and other food products. Although application of chemical fungicides on crops and other food plants ensure significant protection against fungal contamination

(Lagogianni & Tsitsigiannis, 2018), but the transmission of chemical residues in human food chains poses human health at risk (Carvalho, 2017). Another major issue with the irrational use of pesticides is the emergence of resistant fungal strains with higher mycotoxins synthesis potential (Popiel et al., 2017). To address these concerns, biological control achieved through safer microbes (bacteria and yeasts) is getting much popularity to replace synthetic fungicides (Hathout & Aly, 2014; Nešić et al., 2021; Venkatesh & Keller, 2019). The volatiles and diffusible compounds of friendly bacteria and yeasts show significant *in vitro* and *in vivo* fungal growth inhibition and thus mycotoxins synthesis. VOCs of several *Bacillus* spp. are reported to induce antifungal activities by inhibiting fungal growth inhibition, sporulation, and mycotoxins synthesis. On artificially infected coffee beans, Al Attiya et al. (2021) reported significant inhibition of *A. flavus* and *A. niger* by the *B. simplex* BS350-3 volatiles. Likewise, the volatiles of *B. megaterium* 344-1 inhibited the growth of *F. verticillioides, A. flavus*, and *P. verrucosum* (Saleh et al., 2021). Alasmar et al. (2020) and Ul Hassan et al. (2019) reported inhibition of toxigenic *Aspergillus* and *Penicillium* spp., by the volatiles emitted by *Kluyveromyces marxianus* (QKM-4) and *B. licheniformis,* respectively.

This study is designed to explore; (a) the contamination of different date palm fruit varieties with toxigenic fungal species, (b) the occurrence of AFs and OTA produced by these fungi, and (c) the biocontrol potential of *Bacillus cereus* (342-2) on common fungal species of date palm fruit.

2. Material and methods

2.1. Collection of samples and isolation of mycotoxigenic fungi

In total 36 date palm fruits (300–500 g of each) were collected from the supermarkets in Qatar. The samples were categorised into plain ($n = 24$) and stuffed ($n = 12$) with other dry fruits. Stuffed samples were either cashew ($n = 5$), almond ($n = 5$), pistachio ($n = 2$), and almond + pistachio (1). The variety of date, date of collection, packaging, and origin were recorded (Supplementary Table 1).

All date samples ($n = 36$) were surface disinfected using a 1.5% bleach solution for 1 min, rinsed with distilled water twice each time for 1 min, and smashed to paste. In total, each date palm fruit sample (3 g) was mixed with 27 mL of sterile distilled water. For the isolation of fungi, 100 µL of each diluted sample was spread on Rose-Bengal chloramphenicol agar (RBCA) in triplicate Petri plates and incubated at 28°C for 3 days. Thallus forming units (TFU) were calculated as below.

$$Thallus\ forming\ unit\ (TFU) = \frac{(avrg\ NT \times DF)}{plated\ volume} = Thallus/g$$

NT = No. of thalli; DF, dilution factor

All morphologically distinct isolates ($n = 43$) were then purified on duplicate potato dextrose agar media (PDA) using monosporic isolation technique (Balmas et al., 2010). Fungi were incubated at 28°C for 4 days. All media from the growth of fungi and bacteria such as tryptic soy agar (TSA), malt extract dextrose agar (MEA), potato dextrose agar (PDA), Czapek dox agar (CYA) and Rose-Bengal chloramphenicol agar (RBCA) were purchased from Sigma-Aldrich.

2.2. Morphological characteristics of date palm fruit fungi

Two fungal growth media MEA and CYA were used as identification media (Pitt & Hocking, 2009). Purified colonies on PDA were transferred onto triplicate CYA and MEA media plates. For this purpose, fungal spores were suspended in 200 µL of 0.2% soft agar and added with Tween 80 (0.005%). After vortexing briefly, spores were inoculated on 3 spots of the plates using sterile toothpicks and incubated for 7 days (Hassan et al., 2018). Fungal colony diameter, colony colours (observe and reverse), elevation, surface characteristics, sporulation (colour and extent), mycelial colour and density and microscopic observation were recorded and compared with identification reference Pitt and Hocking (2009). All samples were then preserved in 30% glycerol which was prepared by mixing 500 µL of 60% glycerol with 500 µL distilled water in 1 mL Eppendorf tubes. Each colony sample was preserved in 5 replicates and stored at −80°C for later use.

2.3. Molecular identification of toxigenic fungal isolates

In total, 13 fungal strains were selected and further identified on the basis of their molecular profiles using specie-specific PCR primers. This selection was based on their minor morphological differences making them dissimilar from the key characteristics. For this purpose, freshly growing fungal colonies were scraped in liquid Nitrogen and ground to powder. All the steps were followed as described in Qiagen DNeasy plant kit (Hassan et al., 2018). Extracted DNA was ethanol precipitated before further application. PCR reaction mixes were prepared by adding PCR master mix (12.5 µL) obtained from Thermo Fisher Scientific, Waltham, MA, U.S.A., water (9.5 µL), 1 µL of each forward and reverse primer and 1 µL of the extracted DNA. For negative control, instead of a DNA template, water was added. Primers and their annealing temperatures are listed in Supplementary Table 2.

The first PCR reaction was performed using universal ITS1 and ITS4 (Henry et al., 2000) primers to know the suitability of DNA. Annealing temperature for this cycle was at 54°C, and extension temperature at 72°C. The second PCR was done to amplify *A. niger* strains; primers ITS1 forward and NIG reverse were used (González-Salgado et al., 2005). Only black *Aspergillus* samples were tested in this PCR test at an annealing temperature of 66°C. Unamplified black fungi were subjected to another PCR using CAR1/CAR2 primers (Gil-Serna et al., 2009). Primer pair FLA1/FLA2 (Gonzalez-Salgado et al., 2008) was used to amplify DNA samples of *A. flavus* at 58°C annealing temperature. OCRAF/OCRAR primers (Gil-Serna et al., 2009) were used to amplify *A. ochraceus* strains at an annealing temperature of 62°C.

2.4. Quantification of AFs and OTA levels in date palm fruit

Total AFs (including AFB1, B1, G1, and G2) were extracted by mixing ground date samples (5 g) with methanol (25 mL) using the vortex (Al Attiya et al., 2021). After centrifugation, to the 100 µL of supernatant 600 µL of distilled water was added. OTA was extracted by mixing ground date samples (5 g) with diluted Eco solution (25 mL) provided with the kit. To a 500 µL of supernatant 500 µL of buffer solution was added. In each case, 50 µL of diluted extract was added to ELISA wells. Mycotoxins testing

ELISA kits (RIDASCREEN® Aflatoxin total and RIDASCREEN® Ochratoxin A) were obtained from R-Biopharm (Darmstadt, Germany). TECAN Sunrise™ ELISA reader (Männedrof, Switzerland) was used for measuring the absorbance of microplates. Data reduction software (RIDASOFT® WIN, Z9996) of R-Biopharm, Germany was used for the calculation of toxin values of unknown samples. HPLC grade extraction chemicals including dichloromethane (DCM), ethanol, and methanol were purchased from Sigma-Aldrich. In the present work, the limits of detection of AFs and OTA ELISA kits ranged from 0.05 to 4.05 µg/kg (lowest and highest) and 0.03 to 3.00 µg/kg (lowest and highest), respectively. Linearity of ELISA tests for AFs and OTA was 99.72% and 99.84%, respectively.

2.5. Effect of B. cereus 242-2 volatiles on fungal growth

B. cereus 342-2 was isolated from the apricot jam samples (marketed in Qatar), imported from Turkey. Bacterial isolate was identified by Hassan et al., (unpublished data) using Matrix Assisted Laser Time of Flight (MALDI-TOF) mass spectrophotometry (MS). Isolated strain was preserved in 25% glycerol prepared in Luria- Bertani broth at −80°C before retrieving on Nutrient Agar (NA). The volatiles of *B. cereus* 242-2 isolated from apricot jam were used to test their antifungal activity against *Aspergillus* and *Penicillium* species. For this purpose, 100 µL of bacterial cells were incubated for 24 hrs on Tryptic Soy Agar (TSA) plates. On the other hand, 5 µL fungal spores (1×10^4 cells/mL) were placed in the centre of the PDA plate. *A. flavus* (YR18FL), *A. niger* (YR19N), and *A. carbonarius* (YR31C) isolated in this study from the date palm fruit were used in the biocontrol experiments. The choice of these particular isolates for the biocontrol assays was based on their comparative prevalence as well as their mycotoxins in the date palm fruit samples. The covers of both plates were removed, and base plates were sealed against each other (Alasmar et al., 2020). In the control plates, test fungi were exposed to TSA without inoculating any bacteria. At least three 3 replicates of each treated and control were maintained. At day 4 and 7 of co-incubation, fungal colony size was measured and compared with the control. Fungal growth inhibition (%) was calculated for each fungus.

$$Inhibition\ rate\ \% = \frac{(colony\ size\ ofnegative\ control - colony\ size\ of\ treated)}{colony\ size\ of\ negative\ control} \times 100$$

2.6. Identification of bacterial volatiles through GCMS/MS

In order to identify the bioactive antifungal compounds of *B. cereus* 242-2, a setup was prepared for the collection of volatiles. In 250 mL flasks, 100 mL TSB was added, and *B. cereus* 242-2 cells were suspended. The flasks were covered with stoppers where two glass tubes were inserted. One tube was kept 1 cm above the surface of the medium (long tube), while the other at the neck of the flask (short tube). All the connections were tightly sealed, and setup was incubated in a shaking incubator (180 rpm) at 28°C for 24 h. In case of control samples, VOCs were collected from the TSB alone, without bacterial inoculation. Nitrogen gas was introduced from the outer end of the long tube to push the volatiles (produced by bacteria) towards the charcoal trap. VOCs were

eluted using 1 mL dichloromethane (DCM), and transferred to sample collection vials. DCM was injected in GCMS/MS (Agilent 7890 A, CA, U.S.A.) as described by Ul Hassan et al. (2019) and unknown compounds were identified using Wiley and NIST spectral libraries.

2.7. Statistical analysis

Fungal contamination of date palm fruits was presented as % and TFU/g. Mycotoxins contents were presented in ranges (min-max) and compared with the EU permissible limit. For the biocontrol experiments data were analyzed by variance test (ANOVA) and means were compared by post-doc test using Statistical Package for Social Sciences (SPSS) version 23. In all cases, p-values of ≤ 0.05 were kept for any significant difference.

3. Results and discussion

3.1. Occurrence of fungal communities on date palm fruit

Out of 36 date samples, 33 (91.67%) samples were contaminated with different fungal species. Sample with most diverse fungal species was Sayer dates from Doha (sample# 12) with TFU of 15 ± 6.24 thalli/g (Figure 1). This may be because moisture content in this type of dates was found to be 7.5 g/100 g, whereas carbohydrates content is 87.9 g/100 g (Habib & Ibrahim, 2011). The most contaminated sample was sample 14 of Shishi dates from Doha with TFU of 453 ± 6039 colony/g. Moisture level in Shishi dates is known to be 16.31 g/100 g, and carbohydrates at 71.56 g/100 g (Habib & Ibrahim, 2011). Three samples (ID# 30, 34, and 35) of Akhlas dates from Doha showed TFU = 0 colony/g. Moisture and carbohydrates levels in these types of dates are 18.4 and 69.89 g/100 g, respectively (Habib & Ibrahim, 2011). Other possible reasons for the variation in contamination levels among the samples may be due to unsatisfactory storage or packaging techniques for the dates that promote or prevent the fungal growth. Gherbawy et al. (2012) tested 50 date samples from Taif, Saudi Arabia and found 22 fungal species belonging to 12 genera. A high number of fungal isolates showed their toxigenic potential, with 80 *A. flavus* producing aflatoxins and 36 *A. niger* isolates producing ochratoxin. The occurrence of fungal communities in date palm fruit might be associated with the date palm cultivars as reviewed by Abass (2013). A higher frequency of contaminating *Aspergillus* spp., during *in vitro* tissue culture propagation of date palms plants suggests the inherit transfer to fruits. Likewise, Piombo et al. (2020) reported a significant count of contaminating fungal communities in date palm fruits showing a dynamic shift in peel and pulp during different maturation stages.

3.2. Morphological identification of Aspergillus and Penicillium spp.

Isolated fungal strains were subjected to morphological identification using CYA and MEA media (Figure 2). From all the purified samples, 14 different morphological species were isolated. In total, 10 fungal isolates belonging to different species were identified based on their physical and microscopic characteristics. The *Aspergillus* genus was more abundant than *Penicillium*; whereas *A. niger* was the dominating specie among

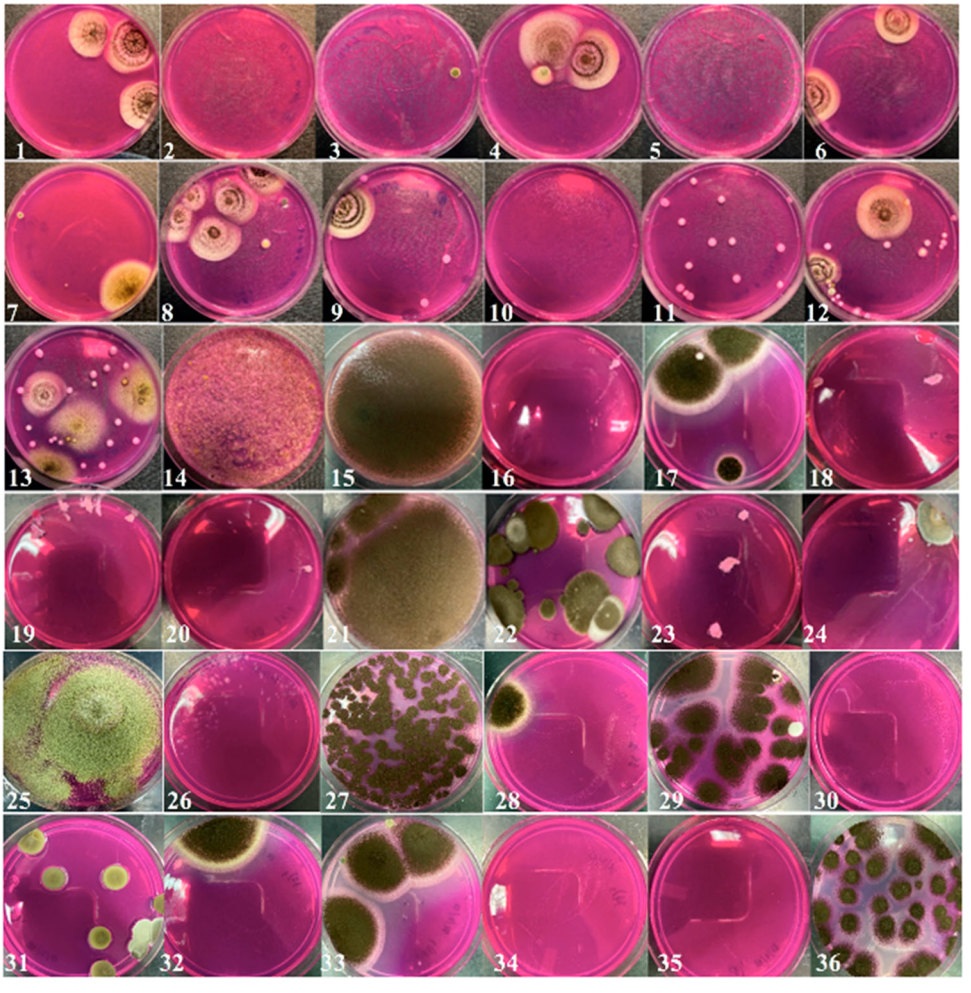

Figure 1. Fungal communities isolated from the date palm fruit samples on RBCA. Diluted samples were plated on the media and colonies were counted at day 3 of incubation at 28°C.

Aspergilli as it was found in 10 samples. The second dominant specie of *Aspergillus* was *A. carbonarius*, which was found in 6 samples. On the other hand, only one species of *Penicillium* (*P. verrucosum*) was found among all the samples. The other identified species were *A. ochraceus, A. parasiticus, A. fumigatus, A. wentii, A. flavus, A. restrictus*, and *A. candidus* (Figure 3). Four fungal species (at least not belonging to ochratoxigenic and aflatoxigenic fungi) were not identified based on their morphological characteristics. High occurrence of *A. niger* in date palm fruits has previously been reported by several researchers. In Nigeria, Colman et al. (2012) found that 39.17% of the date palm fruit isolates were *A. niger*, followed by *A. flavus* (17.60%), *A. fumigatus* (12.5%), and others. In a study conducted by Quaglia et al. (2020) on dried date fruits in Italy, 85% of their dried date samples were contaminated by different fungal genera, including *Aspergillus* (37% of the samples), *Penicillium* (1.8%), *Cladosporium* (0.7%), and other fungal genera after one week of incubation. Moreover, a study conducted in

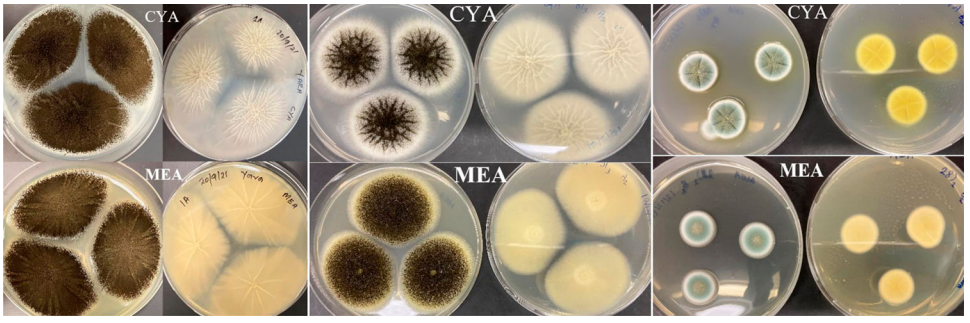

Figure 2. Selected fungal isolates on CYA and MEA. Photos were taken from the observe and reverse side. Samples from left to right are *A. niger, A. carbonarius, and P. verrucosum*.

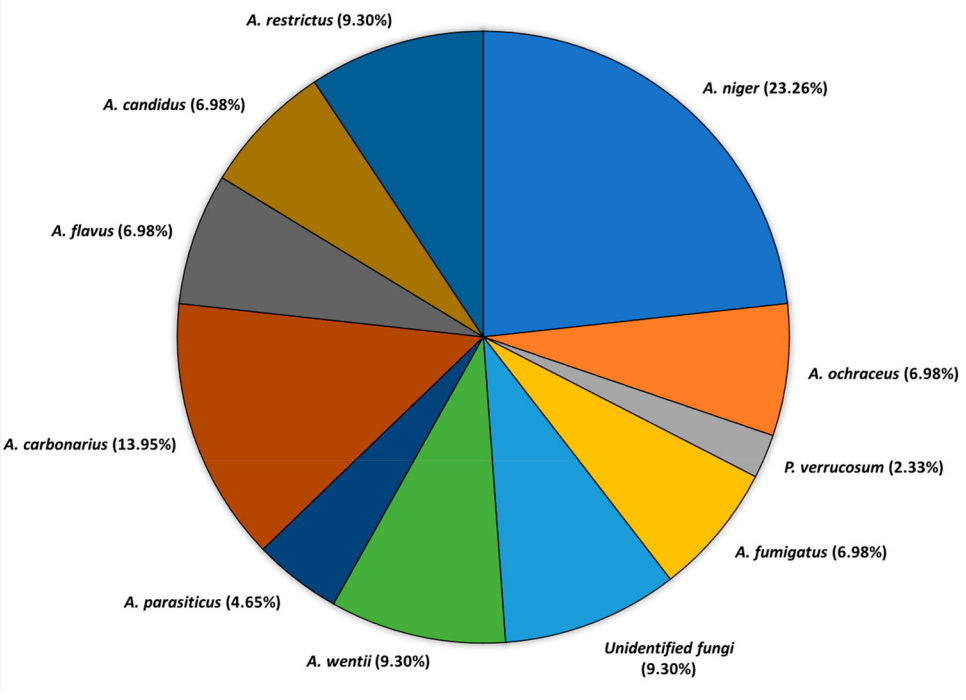

Figure 3. Prevalence (percentage) of fungal species in the date palm fruit samples.

the United Arab Emirates indicates that 40% of the date samples were contaminated by *Aspergillus* toxigenic fungi (Ozer et al., 2012).

3.3. Molecular identification of toxigenic fungi of date palm fruit

Fungal strains displaying minor morphological differences were further characterised using species-specific PCR primers to accurately identify their species. In PCR reactions using ITS1/ITS4 primer pair (White et al., 1990), all fungal DNA samples showed a single band of 550 bp, confirming the suitability of extracted DNA for further amplification

reactions (Figure 4(A)). A specie-specific PCR primer pair (OCRAF/OCRAR) designed by Gil-Serna et al. (2009) for the specific identification of *A. ochraceus* was used, and a single band of 430 bp was obtained (Figure 4(B)). Morphologically, *A. ochraceus* strains display similar characteristics to *A. westerdijkiae* (Pitt & Hocking, 2009), which can accurately differentiate using specie-specific primers as these strains showed no amplification using primers for *A. westerdijkiae*. These results are parallel with the observation of Hassan et al. (2018), where *A. ochraceus* were differentiated from *A. westerdijkiae* using specific primers for two species. PCR amplification results shown in Figure 4C belong to fungal DNA samples amplified using ITS1/NIG primers. This specie-specific primer pair is used to identify *A. niger* (González-Salgado et al., 2005) as well as differentiates *A. carbonarius* from *A. niger* (DNA from

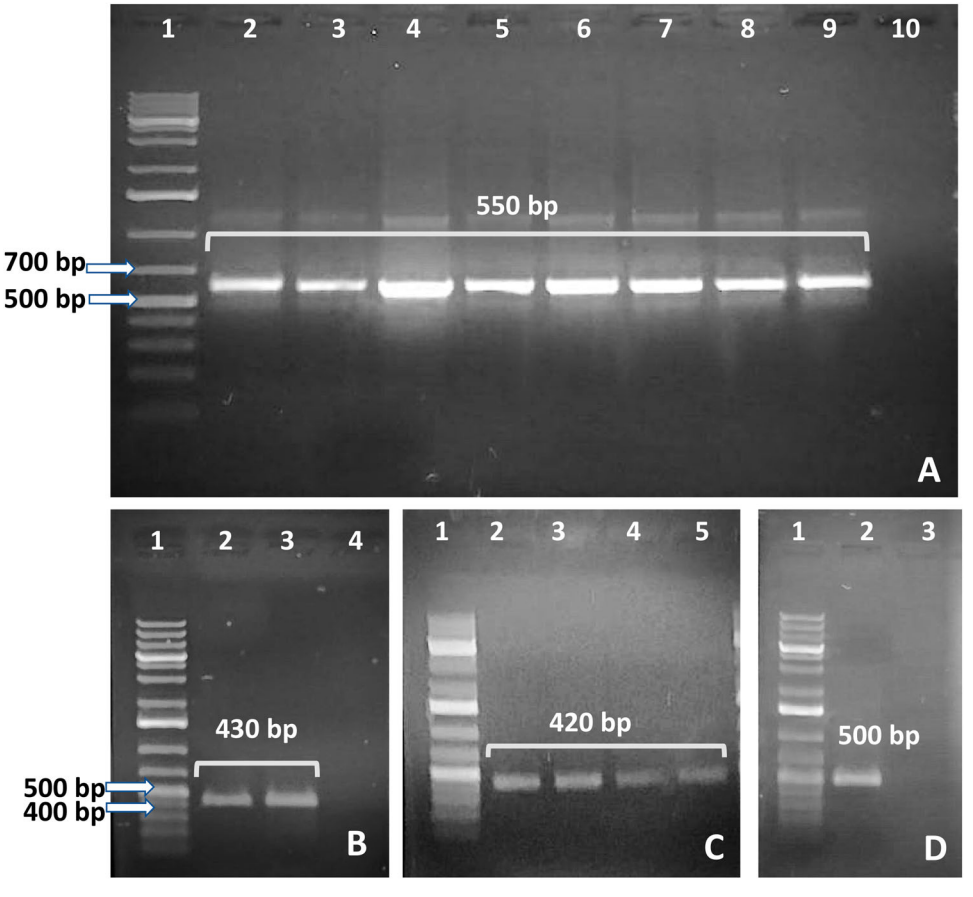

Figure 4. PCR amplification of DNA of the fungal isolates. (A) Lanes; 2–9, primer ITS1/ITS4 with template DNA from *Aspergillus* and *Penicillium* species. Amplicon sizes of lanes 2–9 are 550 bp for all. Lane; 10, negative control (non-template control). (B) Lanes; 2 and 3, primer OCRAF/OCRAR with template DNA from *A. ochraceus,* amplicon sizes 430 bp. (C) Lanes; 2–5, primer ITS1/NIG with template DNA from *A. niger*, amplicon sizes 420 bp. (D) Lanes; 2, primer FLA1/FLA2 with template DNA from *A. flavus*, amplicon size 500 bp. Lanes 1 in all the figures is 1kb plus ladder (20,000 bp, 10,000 bp, 7000 bp, 5000 bp, 4000 bp, 3000 bp, 2000 bp, 1500 bp, 1000 bp, 700 bp, 500 bp, 400 bp, 300 bp, 200 bp, 75 bp).

A. carbonarius is not amplified with this primer pair). In our case, a single band of 420 bp was observed in all 4 samples of black *Aspergilli* using ITS1/NIG primers, suggesting there was no *A. carbonarius* among these isolates. In order to identify and differentiate green *Aspergilli* samples, primer pairs FLA1/FLA2 were used (Gonzalez-Salgado et al., 2008; González-Salgado et al., 2011). PCR amplification results shown in Figure 4D with a single band of 500 bp confirms the presence of *A. flavus*. In line with these findings, Alkuwari et al. (2022) used these specific primers for the identification and differentiation of key toxigenic fungi in cereals including *A. niger, A. flavus, A. carbonarius*, and *A. parasiticus*. Molecular identification of toxigenic fungi is reliable, less laborious and has potential for early identification of fungi to minimise the losses to agriculture and food industry.

3.4. Analysis of AFs and OTA in date palm fruit samples

On the basis of prevalent fungal communities as described in sections 3.1, 3.2, and 3.3 above, the levels of two key mycotoxins (AFs and OTA) were tested in all ($n = 36$) date palm fruit samples. AFs were found in only 2 samples as described in Table 1 at the levels of 1.93 and 1.71 µg/kg. Concentrations of AFs in both samples were below the EU permissible limit of 2 µg/kg. Both samples, i.e. sample# 31 and sample# 32 were stuffed with pistachio and almond, respectively. The occurrence of AFs in the stuffed samples and absence in all plain samples suggest that this mycotoxin might be associated with stuffing material. There are plenty of published evidences on the contamination of date palm fruit samples with AFs (Abdallah et al., 2018; Alghalibi & Shater, 2004; Azaiez et al., 2015; Gherbawy et al., 2012; Luttfullah & Hussain, 2011; Shenasi et al., 2002). Ragab et al. (2001) found aflatoxin B1 (AFB1) in the peanuts' stuffed dates samples at 4.8 and 6.2 µg/kg. In Yemen, much higher levels of AFB1 contamination at 110–180 µg/kg in naturally contaminated dates Alghalibi and Shater (2004). In most of the previous studies the frequency of contaminated samples and levels of AFs were much higher compared with our findings. These differences might be associated with the geographical environmental conditions favouring the distribution and synthesis of AFs. Other factors could be the management of the date palm fruits during post-harvest drying, sorting, packaging, and storage stages.

In our study, OTA was detected in 6 (16.67%) date palm fruit samples all within the EU permissible limit of 4 µg/kg (EC, 2006). Overall, date palm fruits marketed in Qatar are of good quality and safe for human consumption without posing a major risk of mycotoxins exposure to the consumer. In a recent study from Egypt, Abdallah et al. (2018) detected OTA, fumonisins, AFB1, and kojic acid in marketed date fruit samples. In Yemen, Alghalibi and Shater (2004) analyzed date palm fruits and reported no OTA contamination in any of the tested samples. Azaiez et al. (2015) analyzed 75 date

Table 1. AFs and OTA contamination levels in date palm fruit samples.

Parameters	Aflatoxins (AFs)	Ochratoxin A (OTA)
+ve samples (%)	5.6	16.7
Mean ± SD (µg/kg)	1.82 ± 0.16	1.25 ± 0.56
(Min-Max)	(nd – 1.93)	(nd – 2.38)
Samples > EU permissible levels (%)	0	2.78

palm fruit samples collected from Tunisia ($n = 48$) and Spain ($n = 27$) and found OTA in 18 samples at an average level of 1.3 µg/kg (range 0.57–3.3 µg/kg). All OTA-contaminated samples belonged to Tunisian market, while none of the samples collected from the Spanish market was positive for OTA. In the present study, the effect of date palm fruit packaging and origin was not significant on fungal load as well as mycotoxins contents.

3.5. Effect of B. cereus 342-2 volatiles on the growth of fungi

Three toxigenic fungal isolates *A. flavus* (YR18FL), *A. niger* (YR19N), and *A. carbonarius* (YR31C) were exposed to *B. cereus* 342-2 volatile. Fungal growth was monitored by measuring the colony diameter on day 4 and day 7 of co-incubation and compared with non-exposed control fungi. Effect of *B. cereus* 342-2 VOCs was highly significant on *A. niger* (YR19N) and *A. carbonarius* (YR31C) as the bacterial volatiles completely inhibited (Figure 5 and Table 2) their growth till day 7 of co-incubation. However, in case of *A. flavus* (YR18FL), on day 4 colony size of the exposed fungi was significantly smaller (3.53 ± 0.42 mm) compared to the unexposed control (4.03 ± 0.06 mm), whereas on day 7 colony diameter of treated fungi was higher (6.93 ± 1.01) than the control (6.36 ± 0.06). However, until day 7, sporulation in *A. flavus* was completely inhibited by the bacterial volatiles. There are several reports on the inhibition of toxigenic fungi by the volatile molecules produced by *Bacillus* spp., (Al Attiya et al., 2021; Calvo et al., 2020; Chaves-López et al., 2015; Morita et al., 2019; Saleh et al., 2021; Toral et al., 2021; Ul Hassan et al., 2019). In line with the present study, Chaves-López et al.

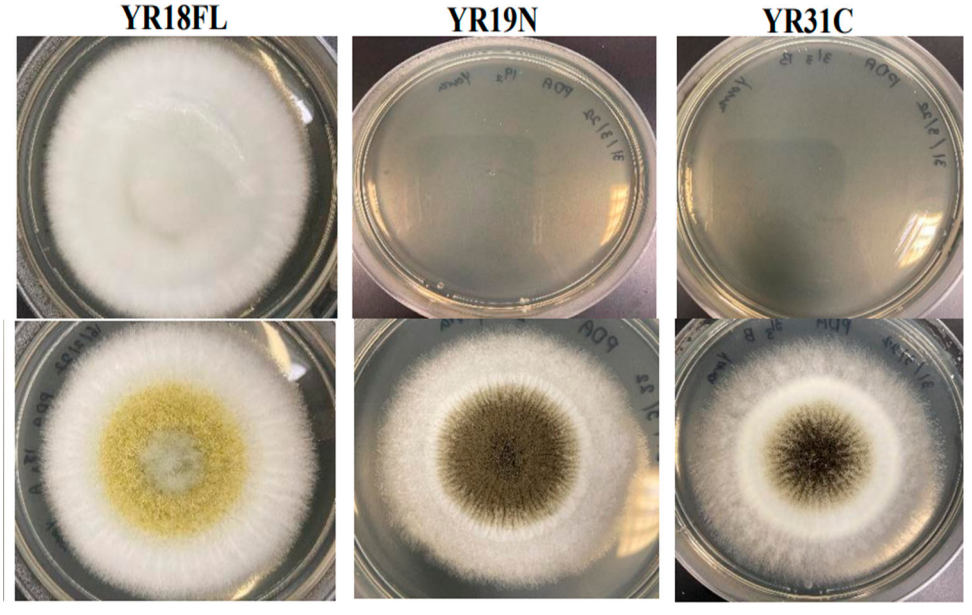

Figure 5. Effect of *B. cereus* 342-2 volatiles on fungal colony diameter. *A. flavus* (YR18FL), *A. niger* (YR19N) and *A. carbonarius* (YR31C) were co-incubated in a co-culture experiment and fungal colony diameters were measured at day 4 and 7.

Table 2. Effect of *B. cereus* 342-2 volatiles on the colony diameter of toxigenic fungi.

	Colony diameter in mm			
	At day 4 of experiment		At day 7 of experiment	
Fungi	Control	Exposed to VOCs	Control	Exposed to VOCs
A. flavus (YR18FL)	4.03 ± 0.06[a]	3.53 ± 0.42[b]	6.36 ± 0.06	6.93 ± 1.01
A. niger (YR19N)	3.83 ± 0.15[a]	0	6.10 ± 0.17[a]	0
A. carbonarius (YR31C)	4.20 ± 0.21[a]	0	6.3 ± 1.42[a]	0

(2015) reported significant growth inhibition of toxigenic *A. parasiticus, A. flavus,* and *A. niger* exposed to volatiles emitted by *B. cereus* and other *Bacillus* spp. Similarly, Fujiu et al. (1994) reported the inhibition of fungal growth by Azoxybacilin produced by *B. cereus* NR2991. In another study, Al Attiya et al. (2021) reported significant inhibition of spread and mycotoxins accumulation by *A. flavus* and *A. niger* on the coffee bean by the volatiles (mainly composed of quinoline,1-Octadecene and benzenemethanamine) of *B. simplex* BS350-3. Likewise, the volatiles mix (hexadecanoic acid methyl ester + tetracosane) of *B. megaterium* 344-1 inhibited the growth of *F. verticillioides, A. flavus*, and *P. verrucosum* (Saleh et al., 2021).

3.6. Composition of B. cereus *342-2 volatiles analyzed by GCMS-MS*

In the recent years, there has been huge work on the exploration of novel bacterial and yeast strains and their molecules agonist of toxigenic and phytopathogenic fungi (Freimoser et al., 2019; Salas et al., 2017; Schmidt et al., 2016). There are several known mechanisms of action of these biocontrol agents, among them are the production of antifungal molecules (volatile and/or diffusible). In the present study, *B. cereus* 342-2 volatiles analyzed by GCMS-MS showed the presence of 1,2-Benzenedicarbonitrile, 2,4-Dimethyl Benzaldehyde, aldehyde, n-Hexadecanoic acid, Undecane, Nonadecane, 1-octanol, Eicosane, and Hexadecane. Lack of such molecules in the head-space volatilome of control tubes showed that antifungal activity of *B. cereus* 342-2 was due to these compounds. In the present study, aldehyde constitutes a major compound in the volatiles mix of *B. cereus* 342-2 with a peak area of 10.60%, followed by 1-octanol (6.30%), n-Hexadecanoic acid (6.08%), and Undecae (2.02%). The antifungal activities of these molecules are already reported in previous studies. For instance, benzaldehyde (Calvo et al., 2020; He et al., 2020; Osaki et al., 2019), n-Hexadecanoic acid (Shehata et al., 2019), and alkanes Undecane (Minerdi et al., 2009; Shehata et al., 2019; Yuan et al., 2012) were detected in the head-space volatiles of antifungal microbial strains. Several studies confirmed the production of alcohols as antagonistic molecules by yeast and bacterial cells. Chaves-López et al. (2015) observed as 1-octanol is one of *Bacillus* volatile compounds previously confirmed to have antifungal activity. Likewise, Zhang et al. (2013) reported that hexadecane produced by *B. atrophaeus* has significant antifungal activity with inhibition rate of 4.7%.

Application of microbial volatiles in the food system including pre-harvest production and post-harvest storage is comparatively new avenue to replace the synthetic pesticides. In this domain, contactless interaction of safe microbes (bacteria, yeast, and fungi) or their antagonistic molecules (volatiles and/or diffusible) is proposed against spoilage fungi, particularly during the post-harvest close-environment storage of food products. In this study, the safety aspect of biocontrol strains (*Bacillus cereus* 342-2) which was

isolated from a food product (apricot jam) is not tested, for this reason can be proposed as safe bio-preservative. However, the antagonistic volatiles emitted by *Bacillus cereus* 342-2 could be used in the food preservative against toxigenic fungi.

4. Conclusion

Dates palm fruit is one of the most consumed dried fruits in the Middle East, and among the favourable substrate for fungal contamination. In this study, 36 date palm fruits of different varieties were used in this study. Except for three, all other 33 samples showed at least one fungal colony. AFs and OTA were found contaminating 5.56% and 16.67% of the date samples, respectively. Levels of AFs and OTA were all below the threshold limits. Toxigenic fungi from *Aspergillus* and *Penicillium* were identified on the basis of their morphological characteristics as well as molecular profiles, however the levels of mycotoxins don't represent any significant threat to public health. VOCs released by *B. cereus* 342-2 significantly inhibited *A. flavus* and completely blocked the growth of *A. niger* and *A. carbonarius*. Further studies based on more varieties and bigger sample size will provide deeper insight to investigate the presence of mycotoxins, toxigenic mycobiota and inhibitory ability of *B. cereus* 342-2 against the wider range of fungal species to minimise the impact of fungal contamination. In the present work, the experimental setup for *Bacillus* volatile analysis was different in the sense that for GCMS-MS analysis head-space VOCs were collected from bacterial culture in TSB in 250 mL flasks, while in case of *in vitro* inhibitory assays bacterial cells were grown in Petri dishes on TSA. Although the nutrients were similar, but the physical state and incubation condition were different, that might induce minor variation in the VOCs profile of antagonistic bacterial strain.

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Disclosure statement

No potential conflict of interest was reported by the author(s)

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