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Investigation and Application of *Bacillus licheniformis* Volatile Compounds for the Biological Control of Toxigenic *Aspergillus* and *Penicillium* spp

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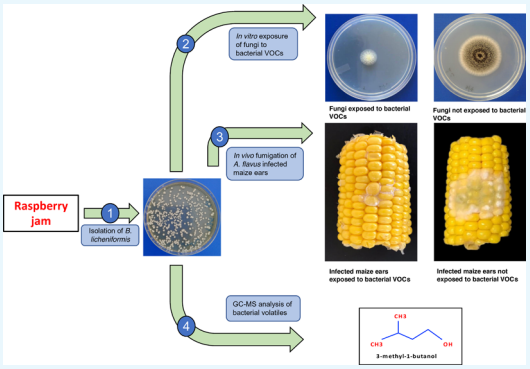
ABSTRACT: The present study was designed to investigate the antagonistic activity of *Bacillus licheniformis* BL350-2 against mycotoxigenic strains of *Aspergillus* and *Penicillium*. In vitro coincubation for 5 days indicated *Aspergillus westerdijkiae* BA1 as the most sensitive strain, with a growth inhibition of 62%, followed by *A. carbonarius* MG7 (60%), *Penicillium verrucosum* MC12 (53%), *A. niger* MC05 (50%), *A. flavus* CM5 (49%), *A. parasiticus* SB01 (47%), and *A. ochraceus* MD1 (44%). Likewise, the majority of the tested strains on exposure to bacterial volatiles showed complete inhibition of mycotoxin synthesis. In vivo assays on maize ears resulted in 88% reduction in *A. flavus* CM5 growth and complete inhibition of fungal sporulation and aflatoxin accumulation. The GC−MS-based volatile profile showed 3-methyl-1-butanol as the most abundant compound. The findings of the present study advocate that *B. licheniformis* BL350-2 is suitable as a biocontrol agent against mycotoxigenic fungi, at least during storage of cereal grains.



INTRODUCTION

Contamination of food and feed with mycotoxigenic fungi and their toxic metabolites is a persistent threat to human and animal health. The ever-increasing list of mycotoxins includes more than 400 bioactive compounds. Aflatoxins (AFs) and ochratoxins (OTs) are known as the most toxic fungal metabolites and are produced by several species of *Aspergillus* and *Penicillium*. *Aspergillus flavus* and *A. parasiticus* are two important *Aspergilli*, responsible for the accumulation of AFs in cereals and other food commodities.[1,2] Aflatoxin B1 (AFB1), among other toxicological properties, presents strong hepatotoxic, immunosuppressive, and carcinogenic activities.[3] The International Agency for the Research on Cancer has classified AFB1 among group-1 human carcinogens.[4] Ochratoxin A (OTA), after AFB1, is another important mycotoxin, with nephrotoxic and possible human carcinogenic effects.[5] The contamination with OTA of food and feed commodities mainly results from the pre- and post-harvest infection by filamentous fungi belonging to the species *A. ochraceus*, *A. westerdijkiae*, *A. carbonarius*, *A. niger*, and *Penicillium verrucosum*.[6]

In most countries, strict regulatory limits for the mycotoxin contamination in food and feed are enforced. The European Union has set a maximum AF limit of 20 μg/kg for complete feedstuff and 4 μg/kg for cereals and processed cereal products used for human consumption. Likewise, for OTA contamination, maximum EU limits for cereals and cereal feed products are 250 μg/kg, while for processed foodstuffs this limit is 3 μg/kg.[7−9]

A key to prevention and control of mycotoxins is the preharvest management of the cereal crops and post-harvest storage of grains, minimizing the favorable conditions (humidity, water activity, temperature) for fungal growth. Field application of synthetic fungicides for the prevention of fungal infections on cereal crops has shown promising outcomes, but their residual transfer to the food chain is being strictly regulated around the globe. Additionally, continuous and improper use of these chemicals might result in unwanted outcomes including the emergence of resistant fungal populations[10] and induction of mycotoxin biosynthesis.[11] To decontaminate the grains from mycotoxins, methods include physical, chemical, and biological treatments.[12] Another approach is the removal of mycotoxins from contaminated food and feed that involves their adsorption on binding substances including biological cell walls or cell-wall-based formulations and activated materials.[13] Apart from binding the target mycotoxins, there is always a risk of depriving the animal subjects from dietary important nutrients and antibiotics, which are sequestered in the gastrointestinal tracts by adsorbents.[14] Also, the binding efficacy of the adsorbent molecule is a function of several

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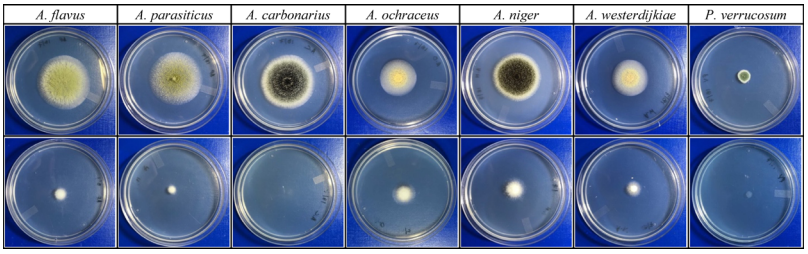

Figure 1. Antifungal activities of *B. licheniformis* 350-2 volatiles against AF- and OTA-producing fungi. The fungi in the first row were not exposed to bacterial volatiles, while those in the second row, showing a significant growth inhibition, were exposed to *B. licheniformis* VOCs. These pictures were taken at day 3 of the experiment (after sealing the plates).

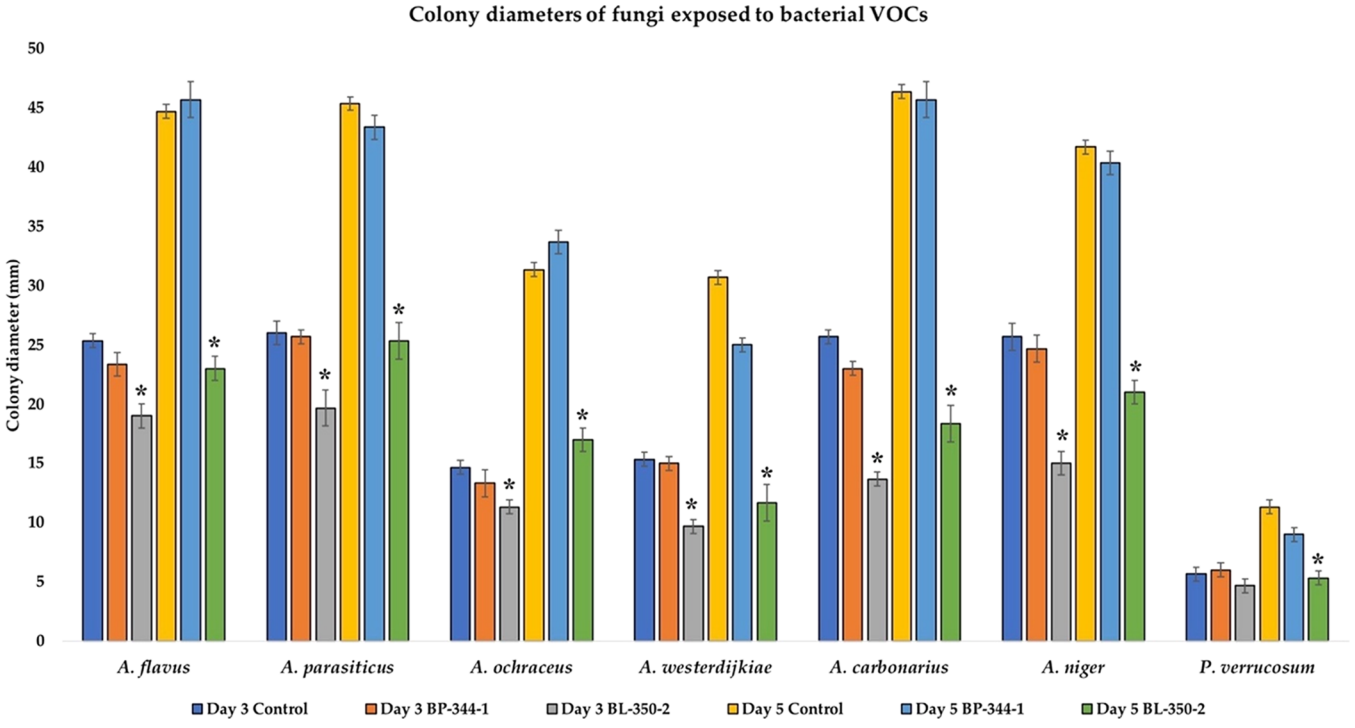

Figure 2. Effect of bacterial VOCs on the fungal colony size (mm). For each time point, measurements were performed on nine fungal colonies. The asterisks indicate the significant ($p < 0.05$) difference between the colony diameter of bacterial VOCs exposed vs unexposed (control) fungi.

factors, including the nature, polarity, size, and concentration of target mycotoxins and especially the pH of the medium.[15] The noncovalent mycotoxin−adsorbent interaction further limits the reliable acceptability of these substances.[16]

In recent years, to address the problem of mycotoxin contamination in food and feed, rigorous focus is being made on the preventive inhibition of fungal growth. To this end, non-pathogenic microbes (yeast and bacteria) are being explored for their potential to be used against toxigenic fungi and their metabolites.[17−19] The commercial efficacies of biocontrol agents against the mycotoxigenic and phytopathogenic fungi are primarily associated with the mode of action of each antagonist.[20] The competition for space and nutrients, induced resistance, production of antifungal compounds (antibiosis), production of antagonistic volatiles, and secretion of enzymes inhibiting the key metabolic processes are potential mechanisms by which bacterial antagonists may inhibit the growth of toxigenic fungi. In these regard, some strains of *Bacillus licheniformis* have also been reported to synthesize the diffusible antimicrobials, which are effective against the important plant pathogenic fungi.[21,22] The aim of this study was to evaluate the potential and nature of the volatiles released/produced by a new antagonistic strain of *B. licheniformis* BL350-2 against the growth and mycotoxin biosynthesis of AF- and OTA-producing *Aspergillus* and *Penicillium* spp.

RESULTS AND DISCUSSION

***B. licheniformis* BL350-2 Inhibits the Growth and Sporulation of Toxigenic *Aspergillus* and *Penicillium* spp.** The application of biocontrol agents, especially bacteria, against phytopathogenic fungi has been established several decades ago. Initially, the antagonism was believed to be the outcome of direct bacterium−fungus interaction, and their mechanisms of action were based on (i) iron depletion of fungi,[28] (ii) degradation of fungal virulence factors,[29] (iii) production of a large variety of antifungal compounds,[30] and/or (iv) induction of systemic resistance.[31] Later, it was realized that some bacteria and yeasts can also exhibit biocontrol activities against fungi from a distance by producing volatiles. For a long time, hydrogen cyanide was the sole known bacterial volatile compound active against pathogenic fungi. Now, several bacterial volatile organic compounds have been explored for their antifungal properties.[18] In the present study,

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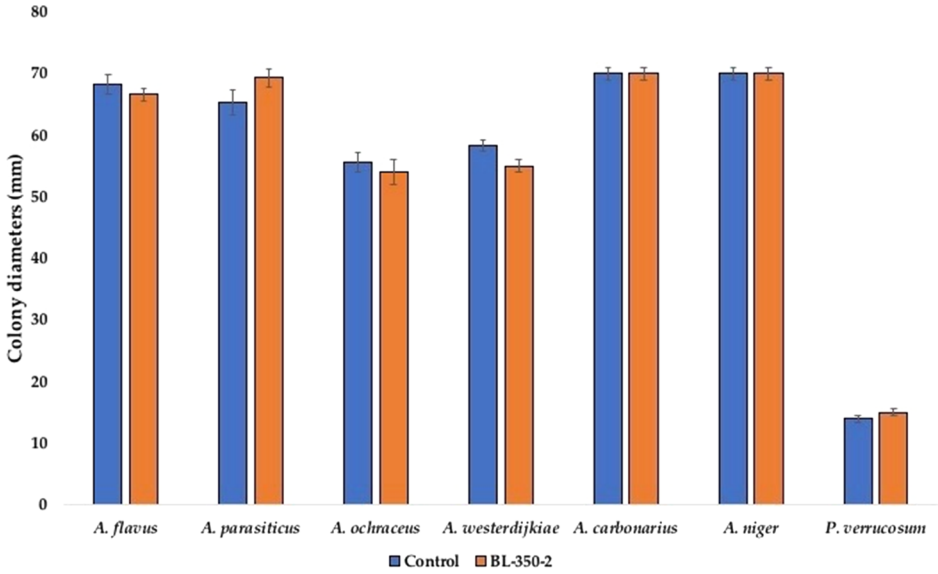

Figure 3. Colony diameters of *B. licheniformis* (BL350-2) exposed fungi on fresh PDA media vs never exposed fungi (control) fungi. Mycelial plugs were removed from the margins of fungal colonies, transferred to fresh PDA, and incubated for 10 days for the measurement of colony diameters (mm). Bars represent the means of nine observations ± SD.

attempts were made to demonstrate the antifungal features of volatiles produced by *B. licheniformis* BL350-2 against aflatoxin- and ochratoxin A (OTA)-producing *Aspergillus* and *Penicillium* species.

The protein profile matching was expressed on the log scale from 0 to 3 score, as per the manufacturer guidelines. The score 1.70−1.999 is designated as probable genus-level identification, and 2.00−2.299 represents confirmed genus-level identification and probable species-level identification. The score 2.30 to 3.0 is highly probable species-level identification. Our bacterial isolate (coded as BL350-2) showed a matching score of 2.32 and was identified as *B. licheniformis*. This score (2.32) falls in the highly probable species-level identification. The test was repeated thrice with the same score of 2.32. In vitro coincubation experiments resulted in a significant reduction of the fungal growth and complete inhibition of sporulation (Figure 1). This result was most likely associated with the volatiles produced by antagonistic *B. licheniformis* BL-350-2 since the experimental setup ensured the lack of physical contact between fungal and bacterial strains. At day 3 of the experiment, except for *P. verrucosum* MC12, all the volatiles-exposed fungal species showed significantly lower colony sizes, as compared to their respective control (Figure 2). However, at day 7 of post-exposure, the colony diameters of all the test fungi, including *P. verrucosum* MC12, were significantly smaller than that of unexposed control (Figure 3).

In general, very low oxygen levels (0.5% or even lower in the environment) are needed to inhibit the fungal growth.[32] In order to rule out the possibility that fungal growth inhibition could be due to accumulation of CO_2 or deficiency of oxygen as reported earlier,[6,33] we coincubated another set of fungi, replacing *B. licheniformis* 350-2 with *B. pumilus* 344-3 (known for not producing antifungal volatiles). In the presence of *B. pumilus* 344-3, there was no effect on fungal growth (Figure 2) and sporulation as compared to control, suggesting that the CO_2 generated or oxygen consumed by bacteria has no effect on fungal growth or sporulation, rather these effects are associated with volatiles produced by *B. licheniformis* 350-2. In the present experiments, either the test fungi were not sensitive to low oxygen tension or the threshold levels were not reached. Additionally, dissolved oxygen concentration in the substrate has a greater impact on fungal growth, rather than atmospheric oxygen.[34]

In line with the present study, a significant reduction in growth, sporulation, and conidial germination of *A. flavus* exposed to volatiles produced by *B. megaterium* KU143, *Microbacterium testaceum* KU313, and *Pseudomonas protegens* AS15 has been reported.[35] In a more recent investigation, the same group of researchers[36] reported that VOCs produced by *B. megaterium* KU143 and *P. protegens* AS15 also exhibit antifungal activities against several other fungi belonging to the genera *Aspergillus* and *Penicillium*. GC−MS-based analysis confirmed that the biocontrol activities were associated with the production of 5-methyl-2-phenyl-1*H*-indole by *B. megaterium* and 2-butyl-1-octanal, dimethyl disulfide, 2-isopropyl-5-methyl-1-heptanol, and 4-trifluoroacetoxyhexadecane by *P. protegens*.[36] Similarly, VOCs produced by *B. subtilis*, *B. amyloliquefaciens*, and *B. cereus* have been reported for their antifungal activities against *Aspergillus*, *Penicillium*, and *Fusarium* spp.[37]

***B. licheniformis* 350-3 Produces Reversible Effects on Fungal Morphology.** The volatiles released by the *B. licheniformis* 350-2 resulted in a significant reduction in growth and complete inhibition of sporulation in all seven tested fungi. However, upon transfer to the fresh PDA media, in the absence of bacterial VOCs, all the fungal isolates displayed normal growth and sporulation, comparable with untreated fungi (results not shown). In line with our findings, the effects of antagonistic yeast *Candida friedrichii* 778 on toxigenic *A. carbonarius* MPVA566 and *A. carbonarius* AN6 were also observed as reversible.[38] The reversibility in the morphological alterations highlights the need for a continuous availability of bacterial VOCs to allow effective biocontrol.

Mycotoxin Biosynthesis Is Inhibited by Bacterial Volatiles. The effects of bacterial VOCs on mycotoxin biosynthesis by toxigenic fungi were quantified at day 10 of the coincubation experiment. The UPLC-based analysis

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confirmed that exposure to *B. licheniformis* 350-2 resulted in a complete inhibition of OTA synthesis by highly toxigenic *A. westerdijkiae* BA1, *A. carbonarius* MG7, and *P. verrucosum* MC12. Nevertheless, in the presence of bacterial volatiles, toxigenic *A. ochraceus* MD1 (21.84 μg/kg) and *A. niger* MC5 (29.32 μg/kg) were still able to synthesize OTA, albeit the levels were significantly lower than that of unexposed control (Table 1).

Table 1. Inhibitory Effect of Bacterial VOCs on the Mycotoxin Synthesis (Mean ± SD) by Toxigenic Fungi[a]

		mycotoxin production (μg/kg)	
fungi	produced mycotoxin	control[b]	VOCs exposed[c]
A. ochraceus MD1	OTA	87.21 ± 6.32[d]	21.84 ± 3.33[e]
A. westerdijkiae BA1	OTA	5141.96 ± 21.41	nd
A. niger MC5	OTA	75.44 ± 7.90[d]	29.32 ± 5.03[e]
A. carbonarius MG7	OTA	941.35 ± 12.54[d]	nd
P. verrucosum MC12	OTA	27.32 ± 2.41[d]	nd
A. flavus CM5	AFs	392.22 ± 15.85[d]	nd
A. parasiticus SB01	AFs	265.34 ± 17.22[d]	nd

[a]Effect of bacterial volatiles on mycotoxin synthesis potential of the toxigenic fungi. Each value represents the mean and SD calculated from the three replicates. The values in each row followed by different superscript letters are significantly different from each other at $p \leq 0.05$. nd − not detected. [b]Mycotoxin contents in the media inoculated with toxigenic fungi. [c]Mycotoxin levels in the media inoculated with fungi as well as exposed to bacterial VOCs.

Similarly, under the influence of bacterial VOCs, *A. flavus* CM5 and *A. parasiticus* SB01 exhibited complete inhibition of AF synthesis, while high levels of toxins were demonstrated by the control cultures. In line with these findings, a significant reduction in AF synthesis by *A. flavus* exposed to VOCs produced by *B. megaterium* KU143, *M. testaceum* KU313, and *P. protegens* AS15 was recorded.[35] The inhibition of mycotoxin production by the toxigenic fungi may be due to bacterial volatiles-associated downregulation in the key genes involved in the mycotoxin biosynthetic pathways in *A. carbonarius* and *A. ochraceus* exposed to yeast VOCs.[23] At low levels of 2-phenylethanol (2PE), a common VOC produced by antagonistic yeast, the AF synthesis inhibition is associated with the stimulation of *A. flavus* growth and decreased breakdown of branched-chain amino acid, which serves as a precursor for the synthesis of AFs.[39]

Bacterial Volatiles Inhibit in Vivo Fungal Infection on Maize Ears. In vivo assays using maize ears were performed in order to investigate the effects of bacterial volatiles on fungal growth and sporulation. Maize ears in the control group (not inoculated with fungal spores) showed no fungal infection. Bacterial volatiles caused a significant ($p \leq 0.05$) reduction in the presence of kernels infected with fungi [7.14 ± 1.97 (mean ± SD)], as compared to high infection percentage (38.77 ± 6.22) in the absence of bacterial VOCs (Figure 4). The presence of TSA media alone had no inhibitory effect on the fungal infection of kernels.

In vivo fungal growth inhibition by *B. licheniformis* BL350-2 is in agreement with what was noted in the in vitro experiments. Our findings are in line with earlier reports of antifungal activities of VOCs produced by *B. amyloliquefaciens* SQR-9 against *Ralstonia solanacearum* infection on tomato roots.[40] Likewise, the volatiles emitted by *B. megaterium* KU143, *M. testaceum* KU313, and *P. protegens* AS15 showed similar activity against *A. flavus*, *A. candidus*, *A. fumigatus*, *P. fellutanum*, and *P. islandicum* infections on stored rice.[35,36]

The AF content in maize kernels was analyzed by UPLC and showed no levels of AF in the untreated kernels and those infected with the *A. flavus* CM5 in the presence of bacterial VOCs. On the other hand, in the absence of bacterial VOCs, *A. flavus* CM5 resulted in the accumulation of high levels of AFs at 173.83 ± 13 μg/kg (mean ± SD) in the infected kernels. The presence of TSA media showed a nonsignificant difference on the AF production potential [181.22 ± 17 (mean ± SD)] of fungi.

3-Methy-1-butanol Is the Main Component of BL350-2 Volatiles. Head space (HS) bacterial volatiles analyzed through GC−MS allowed identification of 32 compounds belonging to different chemical classes, including aldehyde, hydrocarbons, alcohols, and terpenes. In total, 28 compounds detected in the BL350-2 volatiles were also recovered from the TSB media flasks (having no added bacterial) and those inoculated with *B. pumilus* 344-1. The three compounds (benzeneacetaldehyde, 1,5-dimethyl-2-piperidone, and dimethyl disulfide) were produced by the tested bacterial strains, *B. pumilus* 344-1 and BL350-2 (Table 2). The only difference between the volatile profile of *B. pumilus* 344-1 and BL350-1 was the production of 3-methyl-1-butanol by BL350-2, suggesting the responsible compound for its antifungal activity. Recently, the synthesis of 3-methyl-1-butanol by *P. chlororaphis* subsp. *aureofaciens* SPS-41 and its antifungal activity against

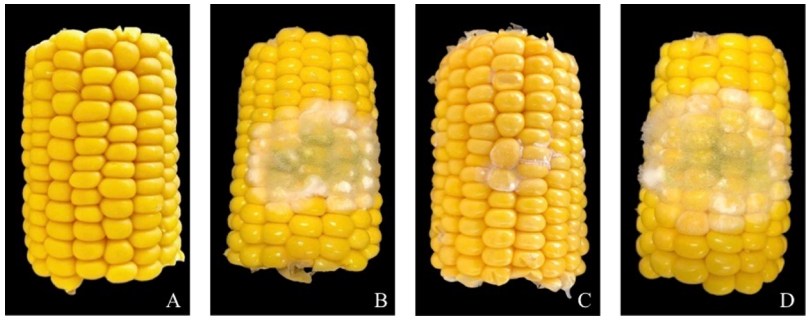

Figure 4. In vivo fungal growth inhibition by bacterial volatiles on maize ears. Control ear-cuts (without fungal spore inoculation) showed no infection (A); meanwhile, *A. flavus* growth was significantly lower on the ear-cuts exposed to bacterial VOCs (C), as compared to fungal infection in the absence of bacteria (D) or in the presence of TSA media (without inoculated bacteria) alone (B).

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Table 2. GC−MS Analysis of Head Space Volatiles of *B. licheniformis* BL350-2 and *B. pumilus* 344-1

S. No.	name[a]	retention time (min)	BL350-2[b] (area %)	BP344-1 (area %)
1	benzeneacetaldehyde	3.327	2.87	3.02
2	1,5-dimethyl-2-piperidone	4.677	5.45	3.22
3	3-methyl-1-butanol	12.422	26.33	1.05
4	dimethyl disulfide	14.102	3.21	2.79

[a]VOCs with a peak area of >1.5% and those not detected in the control (flasks having TSB without any bacterial contamination) are listed in the table. [b]Area (%) of a peak was calculated on the basis of total area of all the detected peaks.

Ceratocystis fimbriata infection in sweet potatoes has been published.[41] Also, some *Bacillus* spp. including *B. subtilis*, *B. amyloliquefaciens*, and *B. cereus* have been reported for the production of 3-methyl-1-butanol, acting as a strong antifungal compound.[37]

In conclusion, a new strain of *B. licheniformis* isolated from raspberry jam and provisionally coded as BL350-2 was tested for its antagonistic activity against representative toxigenic strains of *A. flavus* CM5, *A. parasiticus* SB01, *A. carbonarius* MG7, *A. ochraceus* MD1, *A. westerdijkiae* BA1, *A. niger* MC5, and *P. verrucosum* MC12. In vitro experiments on PDA media showed a significant decrease in fungal growth, sporulation, and mycotoxin biosynthesis of the fungi exposed to bacterial volatiles. Likewise, in in vivo experiments carried out on maize ears, bacterial volatiles resulted in a significant reduction of *A. flavus* CM5 growth and complete inhibition of sporulation and AF biosynthesis. Based on these findings, it can be concluded that the 3-methyl-1-butanol produced by *B. licheniformis* 350-2 confers a significant protection from fungal growth and mycotoxin accumulation, both in vitro and in vivo.

MATERIALS AND METHODS

Strains and Media. All the fungal isolates used in this study were obtained from feed samples, marketed in Qatar. Morphological identification of these isolates was followed by PCR-based identification using species-specific primers. Mycotoxin production potential was tested on yeast extract sucrose (YES) agar, which contained yeast extract (20 g), sucrose (150 g), agar (20 g), and magnesium sulfate (0.5 g) in 1 L of distilled water, and by amplification of genes involved in mycotoxin biosynthesis pathways.[1]

The *B. licheniformis* BL350-2 and *B. pumilus* BP344-1 strains used in this study were isolated from raspberry and strawberry jams, marketed in Qatar (imported from Turkey), respectively. Pure culture was obtained by streaking isolated colonies on Luria−Bertani agar, prepared by adding tryptone (10 g), yeast extract (5 g), NaCl (10 g), and agar (15 g) in 1 L of distilled water.

Identification of Bacterial Strain. Identification of the bacterial strains was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The ethanol/formic acid procedure was adopted for protein extraction. Briefly, a loopful of bacterial cells from colonies grown overnight was suspended in 300 μL of sterile distilled water. After thorough mixing, 900 μL of absolute ethanol was added, mixed, and centrifuged. The supernatant was decanted, and to the pellets, 70 μL of formic acid was added. After thorough mixing by pipetting, 70 μL of acetonitrile was added and centrifuged. A total volume of 1 μL of supernatants was transferred to a MALDI Biotarget sample plate. The spots after drying were overlaid with 1 μL of α-cyanohydroxycinnamic acid (HCCA) matrix solution. The mass-to-charge (m/z) spectrum was obtained by analyzing the samples on a Bruker Biotype (Bruker Daltonics, Bremen, Germany), and identification was made by comparing the spectrum with those available in the database. The protein profile matching was expressed on the log scale from 0 to 3 score, as per the manufacturer guidelines. Before running the test samples, the instrument was calibrated with bacterial test standards provided by the manufacturer (Bruker Daltonics, Bremen, Germany).

Tests for in Vitro Antifungal Activity of *B. licheniformis* BL350-2. The antifungal activity of *B. licheniformis* volatiles was tested by coincubation assays[23] against aflatoxigenic (*A. flavus* CM5 and *A. parasiticus* SB01) and ochratoxigenic (*A. ochraceus* MD1, *A. westerdijkiae* BA1, *A. carbonarius* MG7, *A. niger* MC5, and *P. verrucosum* MC12) fungi. Briefly, from an overnight pure bacterial culture on LB agar, a loopful of cells was suspended in Ringer's solution (0.9% NaCl). To find the best activity of absolute bacterial CFU on the growth and mycotoxin synthesis potential of fungi, serial dilutions of the bacterial cell suspension were made, and from each dilution, 100 μL was evenly spread on tryptic soy agar (TSA) plates, prepared by adding pancreatic digest of casein (15 g), peptic digest of soybean meal (5 g), NaCl (5 g), agar (15 g), K_2HPO_4 (2.5 g), and dextrose (2.5 g) in 1 L of distilled water. This protocol resulted in 12 to more than 500 bacterial CFU/plate of TSA media. These plates were incubated without sealing for 24 h at 30 °C. In the coincubation experiment, the cover of the bacterial plate was replaced by a base PDA plate having inoculated fungal spores, sealed with parafilm, and inoculated. After 3 days of coincubation, we realized that 280−300 bacterial CFU produce enough volatile for the optimal antifungal activity. After optimization, in further experiments, we used only those plates containing 280−300 bacterial CFU each. The lid of bacterial plates was replaced by another base plate, containing a PDA medium point inoculated with 4 μL of fungal spore suspension (10^6 spores/mL). The two base plates were sealed together with four layers of parafilm and one additional layer of sealing tape to prevent VOC escape. *B. pumilus* BP344-1, tested for not producing antifungal volatiles, was used as a negative control. Plates were incubated at 26 °C for 10 days. The effects of bacterial volatiles on fungal growth and sporulation were measured by recording colony diameters and morphology at days 3 and 7 of post-sealing. The control treatment was represented by sealing fungus-inoculated plates with a sterile TSA plate. This experiment was repeated thrice, with six replicates of each test fungi. Each time, three plates of each exposed fungi were opened at day 3 of the experiment (for taking pictures and measuring colony diameters), and the rest were incubated until day 5 of the experiment.

To evaluate the reversibility of the effects on fungal growth and sporulation, at day 5, from the margin of each fungal colony, one plug (~1 cm^2) from each three replicates was removed with a sterile scalpel blade and transferred to fresh PDA plates. These plates were incubated at 26 °C for 7 days. The fungal growth and sporulation were monitored on a daily basis and compared with the control group (never exposed to bacterial volatiles).

In Vivo Antagonistic Activity of *B. licheniformis* 350-2 Volatiles. To test the in vivo antifungal activity of bacterial

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volatiles, full-grown sweet corn (Green Giant, Valencia, Spain) ears were purchased from a local market. Each maize ear was divided in two halves by making a longitudinal cut using a sterile knife. Each half was further divided into three pieces, sterilized with NaOCl (1%) for 2 min, washed twice with sterile distilled water, and dried with absorbent paper.[24] One day before starting the experiment, *B. licheniformis* BL350-2 was streak-inoculated on 90 mm tryptic soy agar (TSA) plates and incubated at 30 °C. Each disinfected maize ear cut was placed on a sterile platform in a glass box (12.5 × 12.5 × 5.5 cm), below which an opened-lid bacteria-inoculated plate was placed to allow the dispersal of volatiles. On the surface of maize ears, 8 μL of the spore suspension of *A. flavus* CM5 (10^5 spores/mL) was placed as a point inoculum. These experiments were repeated thrice with three replicates each time. Appropriate controls were maintained by incubating maize ears, inoculated/uninoculated with fungal spores, in the absence of bacterial VOCs. The containers, upon tight sealing, were placed in an incubator at 26 °C. At day 21 of post-incubation, containers were opened and the percentage of infection was calculated as:

$$\text{infection percentage} = \frac{\text{no. of infected kernels}}{\text{total no. of kernels in maize ear cut}} \times 100 \quad (1)$$

To evaluate the effect of bacterial volatiles on mycotoxin synthesis, 5 g of infected kernels was removed from each replicate and pooled to gather for the analysis of AFs as described below. The mean values of the three experiments were calculated and compared with untreated control.

Mycotoxin Extraction and Analysis. To explore the inhibitory effects of *B. licheniformis* BL350-2 on mycotoxin biosynthesis, another set of experiments having nine fungal plates from each test fungi were exposed to bacterial volatile by the method described above. Control fungi plates were sealed against TSA plates not inoculated with any bacteria. At day 10 of coincubation, colonized media plugs (n = 3/plate) of 7 mm diameter were removed with a sterile cork borer, weighed, and shifted to a 1.5 mL Eppendorf tube.[1] In the case of smaller colony size, plugs from more than one plate of the same fungi were pooled together. Three samples from each fungus were analyzed for either ochratoxin A or aflatoxins (AFs).

OTA was extracted from *A. westerdijkiae* BA1, *A. carbonarius* MG7, *P. verrucosum* MC12, *A. niger* MC05, and *A. ochraceus* MD1 infected media plugs, by adding 1 mL of HPLC-grade methanol (Sigma, St. Louis, MO, USA). The sample was sonicated (Bandelin, Berlin-W. Germany) for 1 h, filtered using 0.22 μm syringe filters (Pall Corporation, MI, USA), and stored at 4 °C until analysis.[25] Before analysis, samples were allowed to evaporate in a SpeedVac and resuspended in the mobile phase. OTA content was analyzed by using an ultra performance liquid chromatography (UPLC) system (Waters, MA, USA) equipped with a Nova-Pak C18 column (4 μm × 3.9 mm × 150 mm) for separation. The isocratic mobile phase was a mix of acetonitrile, water, and acetic acid (45:54:1 v/v/v, respectively). A constant flow rate of 1 mL/min was maintained. A fluorescence detector (FLD) was set at 333 and 460 nm excitation and emission wavelengths, respectively. OTA was identified by comparing the retention time (3.563 min) of the pure OTA reference material (Trilogy Analytical Laboratory Inc., Washington, MO, USA) with a target peak in samples. External calibration using five dilutions of pure OTA standards with a mean correlation coefficient of 0.999626 was used for quantification purposes.

For the quantification of AFs synthesized by *A. flavus* CM5 and *A. parasiticus* SB01, colonized media plugs were dissolved in 1 mL of solvent mixture of methanol, dichloromethane, ethyl acetate (1:2:3 v/v/v, respectively) containing 1% formic acid.[26] After extraction for 60 min, a total of 0.5 mL of sample was transferred to another tube and was dried using a SpeedVac (Thermo Fisher, USA). Before analysis, samples were resuspended in the UPLC mobile phase, which was a mixture of water/methanol/acetonitrile (250/55/55, respectively), and were passed through the syringe filter (0.22 μm). The fluorescence detector was set at 440 and 360 nm emission and excitation wavelengths, respectively. A constant flow rate of 1 mL/min was maintained. Five dilutions of aflatoxin mix standard solution (AFB1, B2, G1, and G2) obtained from Trilogy Analytical Laboratory Inc. (Washington, MO, USA) were used to obtain a calibration curve.

Collection and Analysis of Bacterial Volatiles. The head space VOCs produced by *B. licheniformis* BL350-2 were collected on activated charcoal.[27] For this purpose, BL350-2 was shake-incubated in 200 mL of TSB in 500 mL conical flasks, fitted with two-way rubber corks, with two glass tubes passing through it. To the end of one glass tube, a nitrogen supply system was attached, while its other end was placed just 1 cm above the bacterial culture to remove the head space volatiles. One end of the second tube was placed near the neck of flask, while its other end was attached to a VOC trap. The trap was made of 6 cm long glass tube of 5 mm diameter and filled with 400 mg of activated charcoal (Sigma-Aldrich, Missouri, USA). Before use, the charcoal was wrapped in aluminum paper and placed in an oven at 350 °C for 48 h for sterilization. All the junctions including the neck of the flasks were tightly sealed to avoid the leakage of volatiles. The flasks were placed in a shaking water bath at 30 °C for 48 h. Control flasks contained TSB (alone), and the others were inoculated with *B. pumilus* BP344-1 (not showing antifungal activity). For each treatment, three flasks were maintained for the analysis of VOCs. The nitrogen supply (200 mL/min) was introduced 10 h after inoculation of bacteria. The VOCs on the activated charcoal were eluted with 1 mL of dichloromethane (Sigma-Aldrich, MO, USA) into new glass vials.

Analysis of the volatiles was performed using gas chromatography (GC) with an MSD detector (Agilent, CA, USA). The samples were separated on fused silica column (25 m × 0.2 mm i.d., 0.11 μm). Helium was used as the carrier gas, and the flow rate was maintained at 1 mL/min. Column temperatures were programmed at 30 °C for 3 min and then increased at a rate of 4 °C/min to 210 °C. The mass spectra of the unknown compounds were compared with those of the NIST/EPA/NIH mass spectral libraries.

Statistical Analysis. Data obtained from the in vitro experiments regarding the effects of bacterial VOCs on fungal growth and mycotoxin synthesis potential was analyzed by the analysis of variance test (ANOVA). In the case of in vivo experiments data, if ANOVA showed a significant difference among the treatment groups, post hoc analysis was performed using Fisher's Least Significant Difference test. Means were considered significant at $p \leq 0.05$. SPSS statistical software (Version 23, IBM, NY, USA, 2017) was used for this purpose.

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Notes

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