Current Microbiology (2024) 81:62
https://doi.org/10.1007/s00284-023-03575-8

Bacillus licheniformis M2-7 Decreases Ochratoxin A Concentrations in Coffee Beans During Storage

María Rojas-Pablo[1] · Erubiel Toledo-Hernández[1] · Miguel A. Rodríguez-Barrera[1] · Jeiry Toribio-Jiménez[1] · Carmina Torreblanca-Ramírez[2] · Víctor Manuel Rosas-Guerrero[3] · Sergio Adrián Salgado-Souto[4] · Patricia Álvarez-Fitz[5] · Alejandro Bolaños-Dircio[1] · Yanet Romero-Ramírez[1]

Received: 29 May 2023 / Accepted: 29 November 2023

Abstract

Microbial contamination of coffee beans arises from various factors such as harvesting, handling, and storage practices, during which ochratoxin A (OTA)-producing fungi develop and proliferate. The presence of elevated concentrations of OTA poses a serious health risk to coffee consumers. Therefore, the implementation of a post-harvest treatment involving the use of bacteria known to antagonize OTA-producing fungi constitutes a safe alternative for reducing or eliminating the toxin's concentration in coffee beans. In this study, coffee beans (*Coffea arabica* L.) were inoculated with *Bacillus licheniformis* M2-7, after which we monitored fungal growth, in vitro antagonism, and OTA concentration. Our findings demonstrated that coffee beans inoculated with this bacterial strain exhibited a significant decrease in fungal populations belonging to the genera *Aspergillus* and *Penicillium*, which are known to produce OTA. Moreover, strain M2-7 decreased the growth rates of these fungi from 67.8% to 95.5% ($P < 0.05$). Similarly, inoculation with *B. licheniformis* strain M2-7 effectively reduced the OTA concentration from 24.35 ± 1.61 to 5.52 ± 1.69 µg/kg ($P < 0.05$) in stored coffee beans. These findings suggest that *B. licheniformis* M2-7 holds promise as a potential post-harvest treatment for coffee beans in storage, as it effectively inhibits the proliferation of OTA-producing fungi and lowers the toxin's concentration.

Introduction

Harvesting, handling, and storage practices play a crucial role in ensuring the safety and quality of coffee beans. During the storage phase, fungi capable of producing ochratoxins proliferate inside the coffee beans, predominantly those belonging to the genera *Aspergillus* and *Penicillium* [1]. The Ochratoxin A (OTA) is a toxin found naturally in various food items, including nuts, cereals, alcoholic beverages, and coffee beans [2, 3]. It is important to highlight that, the OTA is a highly stable molecule, that resists high temperatures, the use of cooking or roasting processes for coffee or any other contaminated food does not alter its structure preserving its toxicity [4].

Consumption of coffee contaminated with OTA causes this molecule to be absorbed through the gastrointestinal tract and easily distributed to the blood and tissues [5]. The consumption of foods and beverages contaminated with high levels of OTA causes alterations in cellular respiration, DNA, and protein synthesis, resulting in the production of hydroxy radicals and consequently important repercussions for health [6]. The European Regulatory Commission (EC) [7] has established Regulation Nº 1881/2006, which outlines permissible mycotoxin levels in food, specifying that the allowable limit of OTA in roasted coffee beans must not exceed the threshold of 5 µg/kg [8].

✉ Yanet Romero-Ramírez
yanetromero7@gmail.com

1 Laboratorio de Microbiología Molecular y Biotecnología Ambiental, Facultad de Ciencias Químico-Biológicas, Universidad Autónoma de Guerrero, C. P. 39070 Chilpancingo, Guerrero, Mexico

2 Facultad de Ecología Marina, Universidad Autónoma de Guerrero, Acapulco, Guerrero, Mexico

3 Facultad en Desarrollo Sustentable, Universidad Autónoma de Guerrero, C.P. 40900 Técpan de Galeana, Guerrero, Mexico

4 Escuela Superior de Ciencias de la Tierra, Universidad Autónoma de Guerrero, C.P. 40323 Taxco el viejo, Guerrero, Mexico

5 Facultad de Ciencias Químico-Biológicas, Laboratorio de Toxicología y Salud Ambiental, Universidad Autónoma de Guerrero, C. P. 39070 Chilpancingo, Guerrero, Mexico

Published online: 12 January 2024

The International Agency for Research on Cancer (IARC) [9] has classified OTA under category 2B as a possible human carcinogen. Various methods have been proposed for OTA removal, including the use of organic and inorganic adsorbents, irradiation, high pressure, ultrasonics, ozone treatment, and thermal degradation [8]. However, very few studies have explored biological alternatives to reduce the growth of toxin-producing fungi and OTA production in coffee. De Melo Pereira [10] reported that *Pichia fermentans* LPBYB13 reduces the growth of *Aspergillus westerdijkiae* in coffee beans, thus decreasing OTA concentrations. Similarly, Peromingo [11] found that *Debaryomyces hansenii* reduced OTA concentrations in dry-cured sausages and ham. Furthermore, Ragoubi [12] successfully eliminated mycotoxins from contaminated foods using lactic acid bacteria (LAB). More recently, Nievierowski [13] reported that *Bacillus velezensis* P1 reduces the occurrence of ochratoxins in grapes infected with *Aspergillus carbonarius* during winemaking.

The mechanism through which microorganisms inhibit or diminish the growth of phytopathogenic or OTA-producing fungi primarily involves an antagonistic effect, which is mainly attributed to the production of mycolytic enzymes, antibiotics, siderophores, and hydrocyanic acid [14]. Recent reports indicate that *Bacillus licheniformis* M2-7 acts as an antagonist against phytopathogenic fungi such as *Aspergillus oryzae*, *Colletotrichum* sp., and *Aspergillus niger*, inhibiting their growth by 35–45% [15]. This inhibition by *B. licheniformis* is associated with malformation and damage to the hyphae, causing them to become thinner, fragmented, and less defined compared to the well-defined, elongated, and unswollen hyphae of the fungus when growing in the absence of the bacterium [15].

Additionally, *B. licheniformis* M2-7 has also been evaluated against other phytopathogenic fungi such as *Fusarium oxysporum*, *Microdocum* sp., *Curvularia lunata*, and *Phoma* sp., resulting in growth rate reductions ranging from 22.6% to 81.1% [16]. Based on these previous reports, our study sought to explore a novel strategy to mitigate the proliferation of OTA-producing fungi in coffee beans through post-harvest treatments employing antagonistic microorganisms. Specifically, our study assessed the impact of *B. licheniformis* M2-7 on OTA-producing fungi and the concentration of OTA in Capulin coffee beans during storage. Therefore, the findings of this study provide a valuable basis for the development of strategies and tools for reducing OTA levels in coffee beans under storage conditions.

Materials and Methods

Collection of Coffee Samples

The coffee bean (*Coffea arabica* L.) samples were collected in the "Eden" community (altitude 1469 m above sea level latitude 17°29′41.4 altitude 100°45′56.6) municipality of Tecpan de Galeana in the Guerrero State, Mexico. A total of 3300 g of Capulin coffee were harvested by hand from random coffee plants, transported in a cooler in airtight Ziploc bags, and kept refrigerated in the laboratory of Molecular Microbiology and Environmental Biotechnology at the Autonomous University of Guerrero.

Bacterial Strain and Culture Conditions

All experiments were conducted using the *B. licheniformis* M2-7 strain [17]. Briefly, the bacterial cells were grown in Luria Bertani broth (LB) containing the following components per liter: yeast extract (5 g), peptone (10 g), and sodium chloride (10 g), with a final pH adjusted to 7.5. The cultivation was performed at 35 °C with continuous agitation at 180 rpm for 24 h.

Evaluation of *B. licheniformis* M2-7 Growth of the Bioformulates

To develop the bioformulation, a 6% molasses-based medium was inoculated with *B. licheniformis* M2-7 obtained from a 24-h incubation in LB medium culture. The initial optical density at 600 nm (OD_{600}) was adjusted to 0.02, and the mixture was incubated at 35 °C with continuous agitation at 180 rpm for up to 10 days. To determine the colony forming units (CFU), samples were collected at 0, 3, 6, 12, 24, 24, 36, 48, 48, 60, 72, 96, 120, 144, 168, 192, 216, and 240 h of incubation. The OD_{600} of each sample was then measured, followed by serial dilutions. At the beginning of the experiment (time 0), 50 µl of the culture was extracted and plated. Next, a 1×10^3 dilution was plated at 3 and 6 h, followed by a 1×10^4 dilution from 12 to 240 h. For all dilutions, 50 µl of the culture samples was deposited on nutrient agar plates with the following composition per liter: pluripeptone (5.0 g), meat extract (3.0 g), sodium chloride (8.0 g), and agar (5.0 g). The plates were then allowed to incubate at 35 °C for 24 h and CFUs were counted for each sample in triplicate.

Inoculation of Coffee Beans with *B. licheniformis* M2-7 Bioformulates

Two experimental groups were prepared for the inoculation experiments: the control group (T0), which consisted of 1650 g of coffee beans mixed with a 0.9% saline solution, and treatment 1 (T1), consisting of 1650 g of coffee beans mixed with a bioformulation that had undergone 48 h of incubation (containing 10^8 CFU/ml). Both treatments were incubated for 2 h at room temperature, left to dry for 15 days and then stored for up to 45 days. Throughout this period, samples were collected at 0, 15 and 45 days of storage to

monitor the total fungal colony count and OTA concentration. The total fungal colony count was determined by analyzing four coffee beans from both treatments, T0 and T1, at each of the specified time points (0, 15, and 45 days of storage). The beans were placed in Petri dishes containing PDA medium with the following composition per liter: potato starch (4 g), dextrose (20 g), and agar (15 g). The Petri dishes were then incubated for 7 days at 30 °C, after which colony counting was conducted. The drying process was carried out in an open, ventilated area that facilitated the natural flow of air, allowing the coffee beans to dry at an ambient temperature ranging from 20 to 30 °C, with a relative humidity of approximately 70%, spanning a period of 15 days. The dried coffee beans were then stored in jute bags at room temperature.

Isolation and Macro- and Microscopic Identification of Fungi

Fungal isolation was conducted as described by Luna and Trigos [18]. Briefly, four coffee beans were collected from treatments T0 and T1 at 0, 15 and 45 days of storage. The beans were then placed in Petri dishes containing PDA medium with the following composition per liter: potato starch (4 g), dextrose (20 g), and agar (15 g). Afterward, the Petri dishes were incubated for 7 days at 30 °C, with daily monitoring. For the microculture technique, a humid chamber (Petri dish), and a cube of PDA agar were placed on a slide, then the fungus was seeded on the sides of the agar and a coverslip was placed. After incubation at 30 °C, the coverslip with the formed fungal filaments attached was taken and placed on a slide containing a drop of lactophenol cotton blue for microscopic observation at 100× [19]. The identification of the fungi obtained was carried out by observing the macro and microscopic morphological characteristics with the help of specialized taxonomic keys Barnett and Hunter [20]. Additionally, the Index Fungorum database (http://www.indexfungorum.org/) was consulted. The macroscopic study was determined by observing the color, mycelial growth form and pigment production of each fungal strain. The microscopic characteristics considered were the presence of specialized structures such as conidium, conidiophore, sterigma, metula, microconidium, rhizoid, vesicle, sporangiophore and sporangium.

Molecular Identification of Fungi

Genomic DNA was extracted from fresh mycelia grown in PDA medium and incubated at 30 °C using the Quick-DNATM fungal/bacterial miniprep kit (Zymo research) according to the manufacturer's instructions. The extracted DNA was quantified in NanoDrop Spectrophotometer 2000 (Thermo Fisher Scientific) and analyzed on a 1% (w/v) agarose gel. Next, a fragment (650 bp) of the ITS gene was amplified via polymerase chain reaction (PCR) using the ITS1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-TCCTCCGCTTATTGATATGC-3′) primer pair. Each sample was amplified in triplicate using 10 ng of DNA as a template, 10× reaction buffer (2.5 µl), 25 mM dNTPs (2 µl), $MgCl_2$ (2 µl), 10 pmol oligonucleotides ITS1 and ITS4 (1.5 µl each), and recombinant Taq DNA polymerase (0.5 µl). The volume of each reaction was adjusted to 25 µl using sterile distilled water. The thermal cycler protocol consisted of an initial denaturation cycle at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 59 °C for 1 min, and elongation at 72 °C for 1 min, with a final elongation cycle at 72 °C for 1 min. The resulting amplification products were separated through 1% agarose gel electrophoresis (SIGMA) and visualized using a transilluminator. The samples were then purified using the Gene JET PCR Purification kit (Thermo Scientific).

Sequencing Data and Phylogenetic Analyses

The sequencing was conducted at the Institute of Biotechnology of the National Autonomous University of Mexico (UNAM) using the Sanger method with fluorescent dideoxy terminators and an automated sequencer [21]. The ITS region sequences were aligned and compared with other sequences in Clustal O [22] and GeneBank using NCBI basic local alignment search tools BLAST program [23]. The evolutionary history was inferred using the IQTREE method [24]. The bootstrap consensus tree inferred from 1000 replicates [25] is taken to represent the evolutionary history of the taxa analyzed [25]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [25]. The evolutionary distances were computed using the TIM2 + I + G4 method [26]. Trees were visualized and edited with iTOL v5 [27]. *Saccharomyces pastorianus* as a proper outgroup in the tree was used. The GeneBank accession numbers for the examined ITS region sequences are as follows: *Aspergillus* sp. MCA-4 (OR482198) and *Aspergillus* sp. MCA-5 (OR482199), *Penicillium* sp. MCA-1 (OR482195), *Fusarium* sp. MCA-3 (OR482197) and *Curvularia* sp. MCA-2 (OR482196).

In Vitro Antagonism

To determine the antagonistic effect of *B. licheniformis* M2-7 against fungal growth, in vitro tests were conducted as described by Tejera [28]. The *B. licheniformis* M2-7 inoculum was prepared from a 24-h LB culture incubated at 35 °C. The bacterial culture (10^8 CFU/ml) was then placed in Petri dishes containing PDA medium. A 5 mm diameter disk of mycelium from each fungal isolate was placed in

the center of the Petri dish and incubated for 7–15 days at 30 °C. The diameter of the mycelium was measured with a ruler and the percentage of inhibition was calculated using the equation described by Tejera [28].

$$\text{Inhibitionpercentage}\% = \frac{[(C - T) \times 100]}{C},$$

where C is the diameter of the fungal mycelium in the negative control without bacteria and T is the diameter of the fungal mycelium with *B. licheniformis*.

Determination of OTA

The extraction and quantification of OTA from coffee beans in the T0 and T1 treatments at 0, 15, and 45 days of storage were conducted as described by Estrada [29]. Briefly, 10 g of the ground coffee sample was placed in a 125 ml flask, followed by 50 ml of extraction buffer. The mixture was then centrifuged twice, first for 5 min at 420 rpm and then for 5 min at 3500 rpm, both at room temperature. The OTA concentration was determined using the Ridascreen Fast Ochratoxin test kit (R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions. The absorbance of the samples was measured at 450 nm using a spectrophotometer (Stat Fax 2100).

Statistical Analysis

Statistical analyses were performed using the Prisma and SigmaPlot software. All assays were conducted in triplicate and pairwise comparisons were conducted via Student's *t*-test, whereas the percentage of inhibition was analyzed via Tukey's test.

Results

Bacillus licheniformis M2-7 Growth in the Bioformulations

The growth of *B. licheniformis* M2-7 was evaluated in a molasses-based bioformulation. Figure 1 illustrates the absorbance units (OD_{600}, Fig. 1a), as well as the CFUs (Fig. 1b) obtained from the beginning of the experiment (time 0) to 60 days of incubation. The OD_{600} and the initial CFU concentration of the inoculum in the flasks were 0.02 and 1×10^6 CFU/ml, respectively. The maximum growth was achieved at 60 h of incubation and onwards, with concentrations as high as 10×10^8 CFU with an OD_{600} of 1.04. These results reflect an increase in CFU of 2 units with respect to the initial inoculum.

Bacillus licheniformis M2-7 Reduces Fungal Growth in Stored Coffee Beans

Capulin coffee beans from treatment T0 and treatment T1 were monitored at 0, 15 and 45 days of storage. As depicted in Fig. 2, the CFU count was monitored throughout the observation period. Here, we identified significant differences ($P \leq 0.05$) in the number of CFU in coffee beans without (T0) and with *B. licheniformis* M2-7 (T1) at different storage times (0, 15 and 45 days). Particularly, the T0 coffee beans exhibited a greater number of CFU (Fig. 2a–c), whereas those inoculated with *B. licheniformis* M2-7 (T1) displayed an up to 37.5% reduction in the occurrence of CFU from 0 to 45 days of storage (Fig. 2a–c).

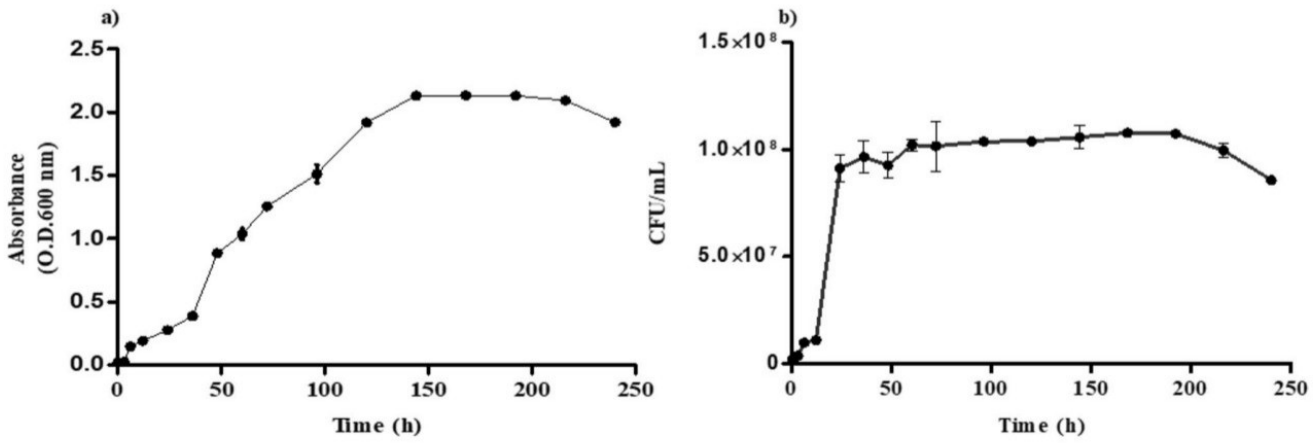

Fig. 1 Growth kinetics of *Bacillus licheniformis* M2-7 in the bioformulation. (**a**) Absorbance units $D.O._{600}$ (**b**) and Colony Forming Units count at 0, 3, 6, 12, 24, 36, 48, 60, 72, 96, 120, 144, 168, 192, 216 and 240 h. The error bars indicate the Standard Deviation (SD) of three replicates ($n = 3$)

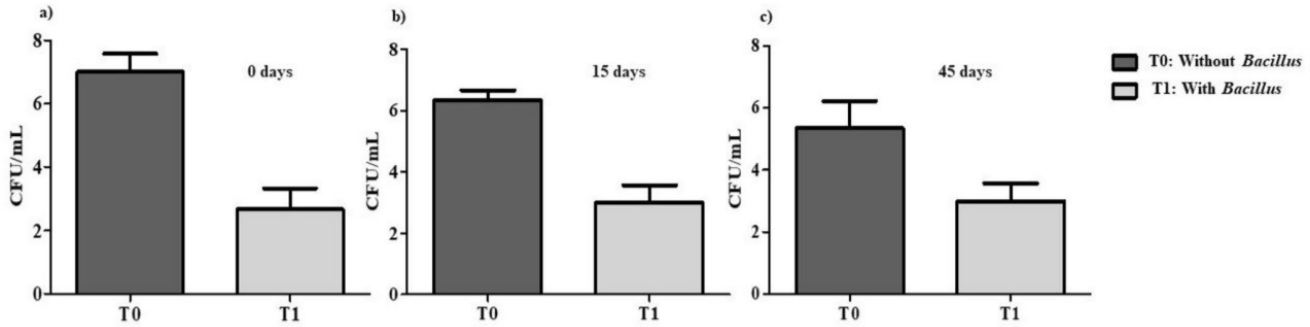

Fig. 2 CFU fungi count from stored dried coffee beans in storage conditions. Coffee beans at 0 (**a**), 15 (**b**) and 45 (**c**) days of storage uninoculated with *B. licheniformis* M2-7 and coffee beans at 0 (**a**), 15 (**b**) and 45 (**c**) days of storage previously inoculated with *B. licheniformis* M2-7. Error bars indicate the Standard Deviation (SD) of three replicates ($n=3$). ***Indicates significant statistical differences ($P<0.05$, Student's *t*-test)

Identification of Two Genera of OTA-Producing Fungi in Stored Coffee Beans

Pure cultures of the contaminating fungi found in Capulin coffee beans (T0) were established for macroscopic and microscopic identification (Fig. 3). Using taxonomic keys and consulting the Index Fungorum database, five fungi belonging to four genera were identified, which were: two *Aspergillus* sp. strains MCA-4 and MCA-5 (Fig. 3a, b, f, g), *Penicillium* sp. MCA-1 (Fig. 3c, h), *Fusarium* sp. MCA-3 (Fig. 3d, i) and *Curvularia* sp. MCA-2 (Fig. 3e, j). Figure 3a, b shows the characteristic macroscopic morphology of *Aspergillus* sp., yellowish green in color. Figure 3f, g shows the microscopic structures conidiophore, vesicle, metula, and conidia are larger and smooth characteristic of the genus *Aspergillus*. Figure 3c shows the macroscopic morphology of *Penicillium* sp., with a woolly or cottony appearance, turning blue-green or gray and growing in 5 days. Figure 3h shows microscopy with septate hyaline hyphae, conidiophores have secondary branches, called metula, long unbranched chains of spores or conidia. Figure 3d shows the macroscopic morphology of *Fusarium* sp., with purple-violet coloration, white cottony mycelium, and growth in 5 days. In Fig. 3i, the microscopy shows elliptical to oval microconidia, single and double chlamydospores, intercalary and terminal, three or four septa, and three or four septa. Figure 3e shows the macroscopic morphology of *Curvularia* sp. growing in 7 days, black hairy color, microscopic morphology shows that it has conidia of cylindrical or slightly curved shape, with one of the central cells being

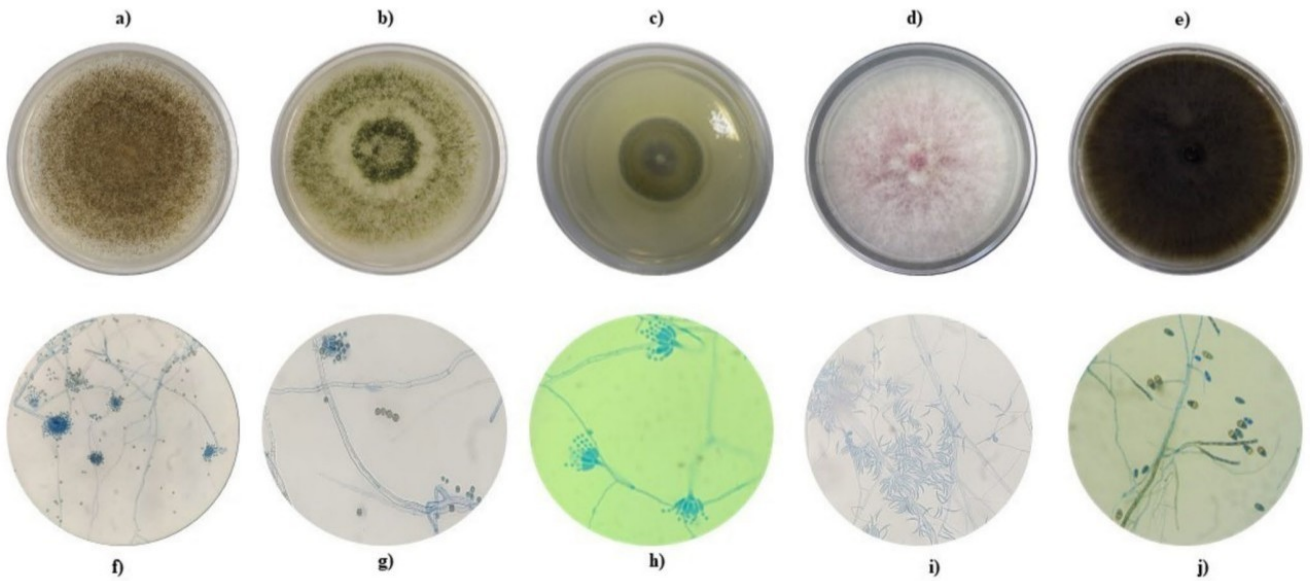

Fig. 3 Macroscopic and microscopic morphology of fungi present in capulin coffee beans. Macro and micro morphology defining to *Aspergillus* sp. (**a**, **b**, **f** and **g**), *Penicillium* sp. (**c** and **h**), *Fusarium* sp. (**d** and **i**), *Curvularia* sp. (**e** and **j**). The microscopic structure was observed at ×100

larger and darker (Fig. 3j). To further characterize the isolated fungi, their ITS region sequences were obtained by amplifying, sequencing their DNA fragments, and analyzing from BLAST. These results were found to be the closest homologs of *Aspergillus* sp. MCA-4, *Aspergillus* sp. MCA-5, *Penicillium* sp. MCA-1, *Fusarium* sp. MCA-3, and *Curvularia* sp. MCA-2 strains (Fig. 4), which coincided with the morphological identification.

The first one belongs to the *Aspergillus* group and contain two isolates showing a unique ITS sequence (MCA-4 and MCA-5) sharing a 94.05% identity with *Aspergillus aflatoxiformans* (NR_171606.1) and *Aspergillus tamarii* (NR_135325.1) with 97.58% identity. The second one (MCA-1) belongs to the *Penicillium* group and displays the same ITS sequence as *Penicillium brocae* (NR_111868.1) with 99.4% identity. The third (MCA-3) belongs to the *Fusarium* group, displaying the same ITS sequence as *Fusarium pseudoanthophilum* (NR_163682.1) with 98.66% identity. The fourth one (MCA-2) belongs to the *Curvularia* group; displaying the same ITS sequence as *Curvularia pseudobrachyspora* (NR_164423.1) with 99.4% identity.

Bacillus licheniformis M2-7 Inhibits the Growth of OTA-Producing Fungi

The impact of *B. licheniformis* M2-7 on the growth of fungi isolated from coffee beans, some of which are known OTA producers (e.g., *Aspergillus* and *Penicillium*), was examined. *B. licheniformis* M2-7 effectively inhibited the growth of all fungi. Specifically, *A. oryzae* MCA-4 growth was inhibited by 74%, *A. tamarii* MCA-5 by 67.8%, *P. brocae* MCA-1 by 87.5%, *Fusarium odoratissimum* MCA-3 by 83.9%, and *C. pseudobrachyspora* MCA-2 by 95.5% (Fig. 5).

Bacillus licheniformis M2-7 Decreases OTA Concentrations in Stored Coffee Beans

The concentration of OTA in Capulin coffee was determined at 0, 15 and 45 days of storage in the absence and presence of *B. licheniformis* M2-7 (Table 1). We observed that the dried coffee beans with 0 days of storage without bacteria (T0) and with *B. licheniformis* M2-7 (T1) exhibited OTA concentrations of 27.76 and 24.35 µg/kg, respectively, with significant differences between the two groups ($P < 0.05$). After 15 days of storage, the dried coffee without bacteria (T0) and with *B. licheniformis* M2-7 (T1) exhibited OTA concentrations of 20.16 and 10.10 µg/kg, respectively. This timeframe exhibited the most significant difference ($P < 0.005$) between the two experimental groups. Coffee stored for 45 days without bacteria (T0) and with *B. licheniformis* M2-7 (T1) exhibited OTA concentrations of 10.45 and 5.52 µg/kg, respectively ($P < 0.05$, Table 1). In dry coffee beans stored for 45 days, the concentration of OTA decreased by 62.35% without *B. licheniformis* M2-7, whereas a 77.33% decrease was achieved in the presence of *B. licheniformis* M2-7.

Discussion

Most studies report that processing conditions play an important role in OTA contamination, since coffee beans are usually dried in a rudimentary manner, exposing them to the environmental conditions of the environment (Dirt, dust and humidity) which favor the growth of OTA-producing fungi [1]. The way to reduce contamination by OTA-producing fungi is by controlling handling conditions [30], but we demonstrate that the development of bacterial formulations and their use as post-harvest treatment, using low-cost raw materials (molasses), is also a good option. Mota et al. [31] reported that molasses is rich in reducing sugars, with approximately 60% of the solids comprising sucrose, glucose, and fructose, in addition to other organic substances such as amino acids that facilitate the biomass development of certain microorganisms. Here, the utilization of a formulation containing *B. licheniformis* M2-7 in a 6% molasses-based medium yielded a favorable growth rate using cost-effective raw materials. The growth of *B. licheniformis* M2-7 in this culture medium can be attributed to the utilization or oxidation of available sugars as energy and carbon sources through catabolic pathways. This phenomenon aligns with the growth exhibited by *Lactobacillus plantarum* when cultivated in a medium containing 20% molasses, reaching concentrations of up to 10^9 CFU/ml [32]. The presence of fungi in coffee beans may be attributed to suboptimal harvesting, processing, and storage practices, along with the prevailing environmental conditions during storage. It is also worth noting that the municipality of Eden experiences a humid and cool climate, with temperatures ranging from 20 to 25 °C. This climate is conducive to fungal proliferation, which is consistent with the findings of Cotty and Jaime [33], who reported that lower temperatures of approximately 25 °C favor fungal growth. Multiple studies have demonstrated the influence of processing conditions on OTA contamination, particularly the use of rustic drying methods that expose coffee beans to environmental factors such as dirt, dust, and humidity, all of which create a favorable environment for the growth of OTA-producing fungi. One common approach to mitigate contamination by OTA-producing fungi is to carefully control handling and processing conditions [30]. However, our findings demonstrated that the development of bacterial formulations and their utilization as post-harvest treatments is also a viable option. This formulation can be effectively used as a post-harvest treatment to reduce the amount of fungi present in coffee beans by up to 37.5% during the storage process. In this work we were able to identify fungi present in coffee beans belonging to the genera *Aspergillus*, *Penicillium*,

Fig. 4 Phylogenetic identification of the fungi based on ITS region of DNA sequences. The evolutionary detachments were calculated using IQTREE method

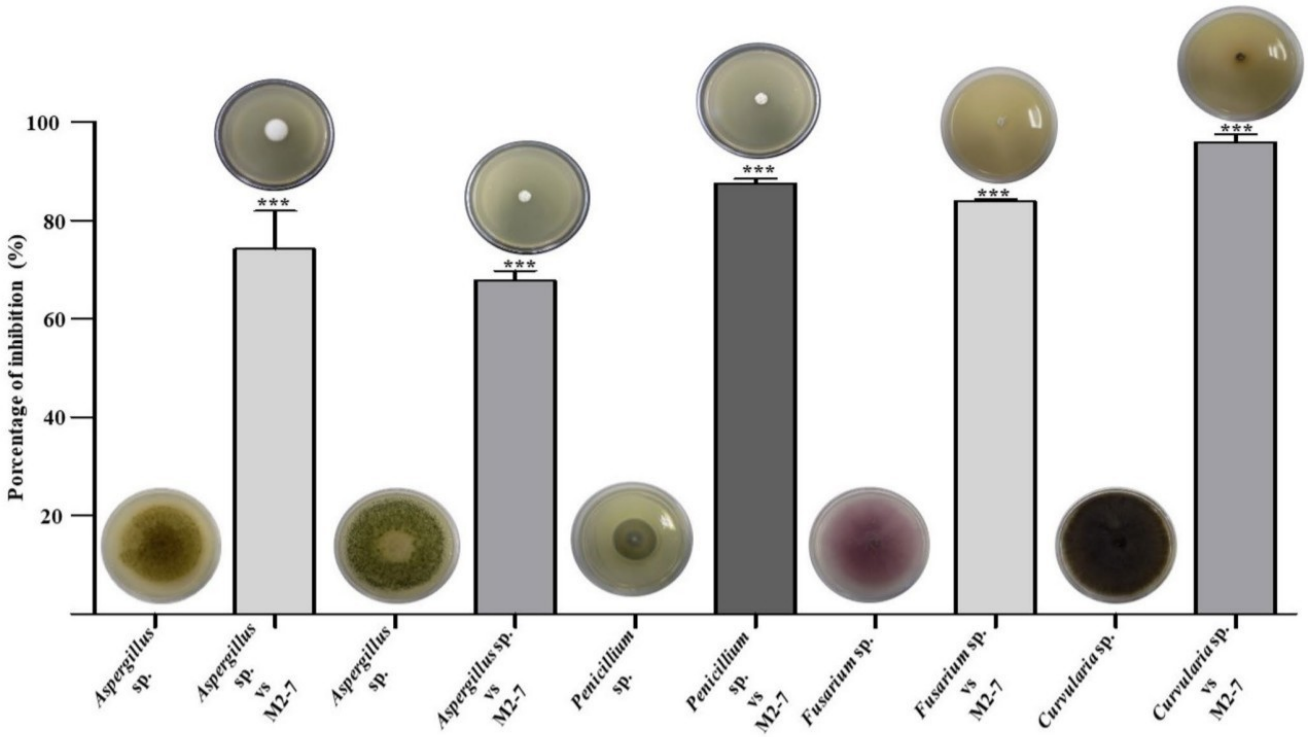

Fig. 5 In vitro antagonistic effect of *B. licheniformis* M2-7 on fungi isolated from coffee beans, *Aspergillus oryzae* MCA-4, *Aspergillus tamarii* MCA-5, *Penicillium brocae* MCA-1, *Fusarium odoratissimum* MCA-3 and *Curvularia pseudobrachyspora* MCA-2. Error bars indicate Standard Deviation (SD) of three independent experiments ($n=3$). ***Indicates significant statistical differences ($P<0.05$, Tukey's test)

Table 1 OTA concentrations of capulin coffee samples at different storage times with no bacteria and with *B. licheniformis* M2-7

Time of storage (days)	Concentration (µg/kg) T0[a]	Concentration (µg/kg) T1[b]
0	27.76 ± 1.12*	24.35 ± 1.61*
15	20.16 ± 1.68**	10.10 ± 0.59**
45	10.45 ± 3.67*	5.52 ± 1.69*

Error bars indicate Standard Deviation (SD) ($n=3$)

[a]T0: Coffee without *B. licheniformis* M2-7

[b]T1: Coffee inoculated with *B. licheniformis* M2-7

* and ** Indicates significant statistical differences ($P<0.05$* and $P<0.005$**, Student's *t*-test)

Fusarium and *Curvularia* by *macro- and microscopic and molecular identification*. The macro- and microscopy identification of *Aspergillus* sp. coincides with that reported by Hu et al. [34], who explain that it is a filamentous fungus with a green color. The identification of *Penicillium* sp. coincides with what was reported by Morales and collaborators [35], who point out that this fungus presents filamentous and hairy bluish-green and gray colonies, with septate hyphae with metulae with long unbranched chains, growth conidia. It is well-known that this fungus causes postharvest fruit rot. The identification of *Fusarium* sp. corresponds to that reported by Castro and collaborators [36], who indicate that present mycelial growth of purple-violet coloration in PDA culture medium with the microscopic characteristics macroconidia, septa, phialides and chlamydospores and is a phytopathogenic fungus of leaves, seeds and fruits. And finally regarding *Curvularia* sp., Requena and collaborators [37] report that it has conidiophores and curved conidia, its growth takes 7 days, its color is black, and it is a phytopathogenic fungus. Among these four identified genera, *Aspergillus* and *Penicillium* have been reported as OTA producers [38, 39]. Wang and Zhuang [40] reported a comprehensive summary of OTA-producing *Aspergillus* species, including *Aspergillus alliaceus*, *Aspergillus sclerotiorum*, *Aspergillus sulphureus*, *Aspergillus albertensis*, *Aspergillus auricomus*, and *Aspergillus wentii*. Similarly, OTA-producing *Penicillium* species such as *Penicillium verrucosum*, *Penicillium nordicum* and *Penicillium expansum* have also been isolated and identified. However, there may still be unexplored species within these genera that possess the capability to produce this mycotoxin. Deoxynivalenol (DON) and T-2 are among the most important mycotoxins produced by the members of the genus *Fusarium*. These mycotoxins are produced during plant infection and, when consumed by humans, can induce intracellular oxidative stress, potentially leading to DNA damage [41]. *Curvularia*, on the other hand,

is primarily recognized as a phytopathogenic agent responsible for seed deterioration and the causative agent of leaf spot disease in maize [42]. The presence of fungi in food poses a serious safety concern, and considerable efforts have been dedicated to inhibiting the growth of microorganism in food. The use of antagonistic bacteria such as *B. licheniformis* M2-7 constitutes an effective strategy is to inhibit the growth of fungi within food matrices [43]. In the present study, inoculating coffee beans with *B. licheniformis* M2-7 substantially decreased the occurrence of fungi during the storage period by impeding their growth. These findings align with those of Bahena-Oregón [16], who reported that the same bacterial strain inhibited the growth of phytopathogenic fungi (e.g., *Microdochium*, *Curvularia*, *Hypoxylon*, *Phoma*, *Xylaria*, *Microphaeropsis*, and *Fusarium*) associated with various diseases in maize, with inhibition rates ranging from 22.6% to 81.1%. Additionally, other studies have indicated that *B. licheniformis* M2-7 inhibited the growth of phytopathogenic fungi such as *A. oryzae*, *Colletotrichum* sp., and *A. niger* by 35–45%. These phytopathogenic fungi are responsible for diseases such as anthracnose and cenicilla in mango crops. The presence of *B. licheniformis* M2-7 causes fragmentation of fungal hyphae and triggers the activation of metacaspases leading to programmed cell death [15]. This mechanism could explain the inhibitory effect exerted by *B. licheniformis* M2-7 against the fungi in this study. It is worth noting that the OTA concentrations in the Capulin coffee used in this study (10.4–27.7 µg/kg) substantially exceeded the permissible levels of OTA established by the European Commission (5 µg/kg). These findings are consistent with those of Álvarez et al. [38], who reported OTA levels in coffee beans from Atoyac de Álvarez, Guerrero, ranging from 6.1 to 93.4 µg/kg. Similarly, Estrada et al. [29] detected high OTA levels ranging from 15.49 to 65.22 µg/kg in coffee beans collected in the State of Guerrero, municipality of Atoyac de Álvarez. Our findings demonstrated that the reduction of OTA in coffee beans inoculated with *B. licheniformis* M2-7 was due to the inhibition of the growth of OTA-producing fungi. However, previous reports have suggested that the microbial load in coffee beans can impact the fermentation process during storage, affecting sensory qualities such as aroma, flavor, body, and acidity [44]. Therefore, additional studies are needed to evaluate whether the presence of *Bacillus* influences the organoleptic characteristics of stored coffee [45].

Conclusions

In this study, we were able to reduce the concentration of OTA in stored coffee beans using a molasses-based bioformulation of *B. licheniformis* M2-7. The mechanism used by the bacterium to decrease this mycotoxin is due to its ability to reduce the growth of OTA-producing fungi. These findings allow consider its application in post-harvest treatments and storage conditions of coffee, which currently are the only strategies to reduce OTA concentrations in coffee through the control of handling conditions. This is due to the application of microorganisms is null and void and has only been reported for sausages, raw hams, and grapes. However, the flavor and aroma qualities of coffee after the implementation of *B. licheniformis* have not been evaluated, which means that these organoleptic qualities should be investigated after the treatment of coffee with this bacterium.

Acknowledgements This work was supported by grants 1056878 from CONACyT. Maria Rojas Pablo thanks CONACyT for PhD scholarships.

Author Contributions All authors contributed to the study conception and design. Investigation and Methodology: MR-P, AB-D and PÁ-F, Data curation: ET-H, Formal Analysis: VMR-G, Supervision: CT-R and SAS-S. The first draft of the manuscript was written by MAR-B and JT-J and Writing—review and editing: YR-R.

Funding This work was supported by grants 1056878 from CONACyT.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethics Approval Not applicable.

References

1. Mondani L, Palumbo R, Tsitsigiannis D, Perdikis D, Mazzoni E, Battilani P (2020) Pest management and ochratoxin A contamination in grapes: a review. Toxins 12(5):303. https://doi.org/10.3390/toxins12050303
2. Chen Y, Chen J, Zhu Q, Wan J (2022) Ochratoxin A in dry-cured ham: OTA-producing fungi, prevalence, detection methods, and biocontrol strategies—a review. Toxins 14(10):693. https://doi.org/10.3390/toxins14100693
3. Ganesan R, Mohan K, Karthick D, Pillay A, Palanisami T, Sathishkumar P, Conterno L (2022) Distribution, toxicity, interactive effects, and detection of ochratoxin and deoxynivalenol in food: a review. Food Chem 378:131978. https://doi.org/10.1016/j.foodchem.2021.131978
4. Van der Stegen H, Essens J, van der Lijn J (2001) Effect of roasting conditions on reduction of ochratoxin A in coffee. J Agric Food Chem 49(10):4713–4715. https://doi.org/10.1021/jf0105586
5. Liu C, Pushparaj K, Meyyazhagan A, Arumugam A, Pappuswamy M, Bhotla K, Baskaran R, Issara U, Balasubramanian B, Mousavi A (2022) Ochratoxin A as an alarming health threat for livestock and human: a review on molecular interactions, mechanism of toxicity, detection, detoxification, and dietary prophylaxis. Toxicon 213:59–75. https://doi.org/10.1016/j.toxicon.2022.04.012
6. Zhai Y, Feng M, Wang W, Yang L, Yang Y (2021) Ochratoxin A: its impact on poultry gut health and microbiota, an overview. Poultry Sci 100:101037. https://doi.org/10.1016/j.psj.2021.101037
7. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in

foodstuffs. http://data.europa.eu/eli/reg/2006/1881/oj. Accessed 1 Jan 2023
8. Leitão L (2019) Occurrence of ochratoxin A in coffee: threads and solutions—a mini review. Beverages 5(2):36. https://doi.org/10.3390/beverages5020036
9. IARC (2016) Agents classified by the IARC Monographs, vols 1–116. http://monographs.iarc.fr/ENG/Classification/. Accessed 25 Oct 2016
10. De Melo P, Beux M, Pagnoncelli G, Soccol T, Rodrigues C, Soccol R (2016) Isolation, selection and evaluation of antagonistic yeasts and lactic acid bacteria against ochratoxigenic fungus *Aspergillus westerdijkiae* on coffee beans. Lett Appl Microbiol 62(1):96–101. https://doi.org/10.1111/lam.12520
11. Peromingo B, Núñez F, Rodríguez A, Alía A, Andrade J (2018) Potential of yeasts isolated from dry-cured ham to control ochratoxin A production in meat models. Int J Food Microbiol 268:73–80. https://doi.org/10.1016/j.ijfoodmicro.2018.01.006
12. Ragoubi C, Quintieri L, Greco D, Mehrez A, Maatouk I, D'Ascanio V, Landoulsi A, Avantaggiato G (2021) Mycotoxin removal by *Lactobacillus* spp. and their application in animal liquid feed. Toxins 13(3):185. https://doi.org/10.3390/toxins13030185
13. Nievierowski H, Veras F, Silveira D, Giocastro B, Aloisi I, Tranchida Q, Dugo P, Brandelli A, Zini A, Welke E (2023) A Bacillus-based biofungicide agent prevents ochratoxins occurrence in grapes and impacts the volatile profile throughout the Chardonnay winemaking stages. Int J Food Microbiol 389:110107. https://doi.org/10.1016/j.ijfoodmicro.2023.110107
14. Das K, Prasanna R, Saxena K (2017) Rhizobia: a potential biocontrol agent for soilborne fungal pathogens. Folia Microbiol 62(5):425–435. https://doi.org/10.1007/s12223-017-0513-z
15. Albarrán-de la Luz L, Rodríguez-Barrera MA, Hernández-Flores G, Lopezaraiza M, Alemán L, Toribio-Jiménez J, Romero-Ramírez Y (2022) Antagonismo of *Bacillus licheniformis* M2-7 against phytopathogen fungi of *Mangifera indica* L. Rev Int Contam Ambie 38:1–10. https://doi.org/10.20937/RICA.54217
16. Bahena-Oregón R, Rodríguez-Barrera MA, Rosas-Guerrero V, Méndez-Bahena A, Toledo-Hernández E, Toribio-Jiménez J, Návez-González D, Palemón-Alberto F, Ortega-Acosta SA, Romero-Ramírez Y (2022) *Bacillus licheniformis* M2-7 inhibits the growth and affects the structure of phytopathogenic fungi of *Zea mays*. In: Sotelo-Navarro PX, Tecorralco-Bobadilla AL, Escamilla-Alvarado C, Hernández-Flores G, Nava-Bravo I, López-Díaz JA, Poggi-Varaldo HM (eds) Ambiente y Bioenergía. Perspectivas y avances de la sostenibilidad. ABIAERA C, México City, México, pp 500–508
17. Guavara J, Serrano I, Rodríguez M, Hernández G, Toribio J, Toledo E, Romero Y (2022) Isolated bacteria from hot springs able to use hydrocarbons as carbon source. Rev Int Contam Ambient 38:68–77. https://doi.org/10.20937/RICA.54234
18. Luna Y, Trigos Á (2010) Aislamiento de cepas de *Aspergillus niger*, productoras de ocratoxina A, en café verde (*Coffea arábica*) almacenado. Rev Mex Micol 32:63–68
19. Riddell W (1950) Permanent stained mycological preparations obtained by slide culture. Mycologic 42:265–270. https://doi.org/10.2307/3755439
20. Barnett L, Hunter B (1998) Illustrated genera of imperfect fungi, 4th edn. APS Press, The American Phytopathological Society, St Paul, MN, p 218
21. Sanger F, Nicklen S, Coulson AR (1977) Sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 74:5463–5467. https://doi.org/10.1073/pnas.74.12.5463
22. Sievers F, Higgins DG (2018) Clustal Omega for making accurate alignments of many protein sequences. Protein Sci 27:135–145
23. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Rapp BA, Wheeler DL (2000) GenBank. Nucleic Acids Res 28(1):15–18. https://doi.org/10.1093/nar/28.1.15
24. Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R (2020) IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol Biol Evol 37(5):1530–1534. https://doi.org/10.1093/molbev/msaa015
25. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
26. Tajima F, Nei M (1984) Estimation of evolutionary distance between nucleotide sequences. Mol Biol Evol 1:269–285
27. Letunic I, Bork P (2021) Interactive tree of life (iTOL) v5: an online tool for phylo-genetic tree display and annotation. Nucleic Acids Res 49:W293–W296. https://doi.org/10.1093/nar/gkab301
28. Tejera M, Rojas M (2012) Antagonismo de *Bacillus* spp. frente a hongos fitopatógenos del cultivo del arroz (*Oryza sativa* L.). Rev Prot Veg 27(2):117–122
29. Estrada B, Salazar R, Ramirez M, Moreno JJ, Romero Y, González M, Alvarez P (2022) Influence of water activity on physical properties, fungal growth, and ochratoxin A production in dry cherries and green-coffee beans. J Food Process Preserv 46:16226. https://doi.org/10.1111/jfpp.16226
30. Pleadin J, Frece J, Markov K (2019) Mycotoxins in food and feed. Adv Food Nutr Res 89:297–345. https://doi.org/10.1016/bs.afnr.2019.02.007
31. Mota L, Guadarrama S, Salas R, Escalante A, Montville Valadez R (2019) Adaptación de *Bacillus licheniformis* a Melazas para la Producción Mejorada de una Cepa Biofertilizante. Agrociencia 53(8):1183–1201
32. Ossa A, Vanegas C, Badillo M (2010) Evaluación de la melaza de caña como sustrato para el crecimiento de *Lactobacillus plantarum*. Rev UDCA Act Div Cient 13(1):97–104
33. Cotty J, Jaime R (2007) Influences of climate on aflatoxin producing fungi and aflatoxin contamination. Int J Food Microbiol 119(1–2):109–115. https://doi.org/10.1016/j.ijfoodmicro.2007.07.060
34. Hu L, Van J, Gruben S, Wösten B, Gu JD, De Vries P (2011) Improved enzyme production by co-cultivation of *Aspergillus* and *Aspergillus oryzae* and with other fungi. Int Biodeterior Biodegrad 65:248–252. https://doi.org/10.1016/j.ibiod.2010.11.008
35. Morales Y, Hernández J, Ramos G, Pérez R, Muñoz R, Muñoz J (2016) Cuantificación de *Penicillium* sp. por el método de goteo en placa. Rev Iberoam Cienc 3(2):13–19
36. Castro O, Kenneth Ramirez A, Blanco MM (2018) Morphological and molecular characterization of *Fusarium oxysporum* f. sp. Apii associated with celery wilt in Costa Rica. Costa Rican Agron 42(1):115–126. https://doi.org/10.15517/rac.v42i1.32199
37. Requena S, Morales P, Gómez S, Boga A, Peláez T (2020) Micosis cutánea por *Curvularia pallescens* en un paciente trasplantado pulmonar: primer caso descrito en España. Rev Iberoam Micol 37(2):58–62. https://doi.org/10.1016/j.riam.2020.03.005
38. Álvarez P, Ramírez R, López L, Moreno MaE (2016) Micotoxinas en Guerrero: presencia de Ocratoxina A en granos de café de Atoyac de Álvarez. Rev Simul Lab 2:12–16 (**ISSN: 2410-3462**)
39. Perrone G, Susca A (2017) *Penicillium* species and their associated mycotoxins. Methods Mol Biol (Clifton, NJ) 1542:107–119. https://doi.org/10.1007/978-1-4939-6707-0_5
40. Wang L, Zhuang WY (2007) Phylogenetic analyses of *penicillia* based on partial calmodulin gene sequences. Biosystems 88:113–126. https://doi.org/10.1016/j.biosystems.2006.04.008
41. Pierzgalski A, Bryła M, Kanabus J, Modrzewska M, Podolska G (2021) Updated review of the toxicity of selected fusarium toxins and their modified forms. Toxins 13(11):768. https://doi.org/10.3390/toxins13110768

42. Wang S, Lu Z, Lang B, Wang X, Li Y, Chen J (2022) *Curvularia lunata* and Curvularia leaf spot of maize in China. ACS Omega 7(51):47462–47470. https://doi.org/10.1021/acsomega.2c03013
43. Khruengsai S, Pripdeevech P, Tanapichatsakul C, Srisuwannapa C, D'Souza E, Panuwet P (2021) Antifungal properties of volatile organic compounds produced by *Daldinia eschscholtzii* MFLUCC 19-0493 isolated from *Barleria prionitis* leaves against *Colletotrichum acutatum* and its post-harvest infections on strawberry fruits. PeerJ 9:e11242. https://doi.org/10.7717/peerj.11242
44. Bressani P, Martínez J, Sarmento B, Borém M, Schwan F (2020) Organic acids produced during fermentation and sensory perception in specialty coffee using yeast starter culture. Food Res Int 128:108773. https://doi.org/10.1016/j.foodres.2019.108773
45. Rojas-Pablo M, Romero-Ramírez Y (2022) El Café te Mejora la Vida, pero sin Ocratoxina. Bol SCME 2(4):80–87