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Investigation of heat-resistant antifungal agents from *Bacillus amyloliquefaciens* and *Bacillus subtilis* for biocontrol of mycotoxigenic fungi

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ABSTRACT

Fungal infections of food and feed with toxigenic strains results in the accumulation of harmful metabolites. In this study, the antifungal activity of two indigenous bacterial strains *Bacillus amyloliquefaciens* and *Bacillus subtilis* isolated from camel feed was investigated. The potential of the bacterial cell free supernatant (CFS) to reduce mycotoxins synthesis was evaluated, and the impact of their antifungal metabolites on the fungal hyphae was examined. High antagonistic activity was exhibited by both bacterial strains. The best activity was observed against two mycotoxigenic *A. niger* strains (Sp31 and Sp33) with inhibition zones of 36.75 ± 0.7 mm and 35.75 ±1.5 mm, by strains *B. amyloliquefaciens* and *B. subtilis,* respectively. The effects of these bacterial strains' CFSs on mycotoxins synthesis were investigated, and both CFSs were found to reduce the mycotoxins synthesis by *A. parasiticus, A. niger* and *Penicillium* spp., in a dose-dependent manner. Moreover, the fungal cell wall morphology was significantly altered by the bacterial CFS at a very low concentration of 1 %. The heat stability of the antifungal metabolites was evaluated at various temperatures ranging from −20 °C to 100 °C, and the metabolites were found to be highly stable, retaining their antifungal activity against *A. niger*. The antifungal activity of *B. amyloliquefaciens* and *B. subtilis* was stable at −20 °C for 236 and 34 days of storage, respectively. These findings suggest that both bacterial strains are exceptional candidates in the biocontrol of mycotoxigenic fungi and in the reduction of their toxic metabolites.

1. Introduction

Mycotoxins are low-weight molecules synthesized as secondary metabolites by mycotoxigenic fungi and are frequently reported in agricultural products (Ferrara et al., 2022; Gurikar et al., 2023). Mycotoxins contaminating food and feed are mainly produced by species of *Aspergillus, Fusarium, Penicillium* and *Alternaria* genera (EL Houssni et al., 2023; Rocha-Miranda and Venâncio, 2019). To date, over 400 mycotoxins have been identified and categorized as toxigenic, leading to adverse health effects such as neurological disorders, hepatotoxicity, and nephrotoxicity (Hamad et al., 2023). Mycotoxins exposure to animals happens with the ingestion of contaminated feedstuff and affects their wellbeing, productivity and may lead to their death (Buszewska-Forajta, 2020; Zeidan et al.,

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2022). Apart from that, the presence of mycotoxins in food and feedstuff reduces the nutrients availability (Luo et al., 2018).

Aflatoxins (AFs) and ochratoxin A (OTA) are the most potent mycotoxins (Mukhtar et al., 2023). AFs can be found in many food commodities including milk, grains, and nuts (Chhaya et al., 2023; Hassan et al., 2018; Osaili et al., 2023; Zeidan et al., 2022), while OTA can be detected in coffee, grapes, wine and cereals (Aguilar-Alvarez et al., 2021; Freire et al., 2020; Tabarani et al., 2020; Zhang et al., 2022). The stability of mycotoxins to physical parameters such as high temperature and pH enables them to persist within food particles throughout processing stages (Marins-Gonçalves et al., 2023). Because of these factors, ensuring food safety and implementing effective control measures are seen as urgent priorities.

Various biological, physical, and chemical treatments are available to mitigate the occurrence of mycotoxins and their residues in food. It is important to note that many of the aforementioned methods can alter the nutritional content and properties of food, affecting its taste and causing the breakdown of vitamins (Mukhtar et al., 2023; Tiwari and Dubey, 2023) Therefore, research has focused on developing various novel methods in response to increasing consumer awareness of food safety. These methods aim to prevent mycotoxin contamination and reduce their residues in food products, while minimizing the impact on food quality.

Some of the emerging techniques include detoxifying food by adding safe bacteria like lactic acid bacteria (LAB), eliminating fungal toxins using natural phenolic compounds, and utilization of natural plants extracts. In biological control strategies, the utilization of bacteria belonging to the *Bacillus* genus is regarded as one of the intriguing options (Lu et al., 2022; Petrova et al., 2022; Saricaoglu et al., 2023). The characteristics that render the *Bacillus* genus suitable for industrial application include the majority of its species being Generally Recognized as Safe (GRAS), their resilience to environmental stressors, genetic stability, and the capacity to form endospores (Hashem et al., 2019; Su et al., 2020).

The three species that are primarily used in commercial biofungicides synthesis are *B. amyloliquefaciens, B. subtilis, and B. velezensis.* (Rabbee et al., 2023). *B. amyloliquefaciens* and *B. subtilis* are considered among the most investigated *Bacillus* strains with regard to their antifungal and antimicrobial characteristics (Bouchard-Rochette et al., 2022; Liu et al., 2019).

Bacillus bacterial species are recognized for producing antifungal metabolites, including cyclic lipopeptides like iturin, fengycin, and surfactin families, as well as polyketides, siderophores, and volatile compounds (Jin et al., 2022; Kang et al., 2020; Higazy et al., 2021; Zeidan et al., 2018).

In the present work, we investigated the antifungal properties of two *Bacillus* strains isolated from animal feed against mycotoxigenic fungi. Considering that OTA and AFs are among the most prevalent mycotoxins in stored food and feed, our goal was to examine how the bacterial cell-free supernatant (CFS) affects fungal growth and the reduction of these mycotoxins. The CFSs were considered as the source of the mixture of antifungal metabolites. Their impact on the morphology of fungal hyphae was assessed using scanning electron microscopy (SEM). Additionally, the thermal stability and storage of the CFSs were evaluated.

2. Materials and methods

2.1. Chemicals

Glycerol, Formic acid (36 %), Acetonitrile (⩾99.9 %), chloramphenicol, PBS (10X), glutaraldehyde (50 %), ethanol (99.8 %) and sodium chloride (NaCl) used in this study were all analytical grades and obtained from Sigma. Nutrient Agar (NA) was prepared by adding beef extract (0.3 %), peptone (0.5 %), sodium chloride (0.5 %) and agar (1.5 %). Lysogeny broth (LB) was prepared in by mixing tryptone (1 %), yeast extract (0.5 %), sodium chloride (1 %) in sterile distilled water. Potato Dextrose Agar (PDA) was made by adding potato dextrose broth (2.4 %) and agar (1.5 %) in distilled media. For the analysis of mycotoxins, Aflatoxins Total ELISA kit and Ochratoxin A 30/15 ELISA kits manufactured by R-Biopharm AG, Darmstadt, Germany were used.

2.2. Isolation and handling of the bacterial strains

Bacterial strains were isolated from camel feed samples following the protocol of Hassan et al., (2019). Wheat bran and mixed feed were collected from Qatari private farms (Zeidan et al., 2022). Briefly, samples of feed were ground into fine powder, and 2 g of powder were added to 30 ml autoclaved dH_2O flasks and were shaken at 30 °C for 5 h. The flasks were allowed to settle and the water on top was collected, diluted and spread on LB agar. Colonies with different appearance were purified and screened for their antifungal activity. Bacteria with antifungal activity were preserved in 30 % glycerol and revived on nutrient agar for further experimentation.

2.3. Bacterial strains identification by MALDI-TOF MS

The identification of the bacterial strains was carried out based on their protein profiles as described by Wang et al. (2012). Fresh 24 h cultures of the bacterial strains were obtained and a bacterial suspension in dH_2O was prepared. Absolute ethanol was added to the suspensions, and they were vortexed thoroughly. The tubes were centrifuged twice at 13,000 rpm for 2 min and the pellet was discarded. Formic acid (70 %) was added to the pellet and the tubes were vortexed. Acetonitrile (50 µl) was added, and the tubes were mixed and centrifuged. 1 µl of the suspension was loaded in duplicate on the Biotarget MALDI-TOF plate. The samples were covered with 1 µl HCCA matrix solution (α-cyano-4-hydroxycinnamic acid). The plate was air dried and then loaded in the MALDI-TOF reader for obtaining protein profiles to be compared with reference peaks in the existing libraries. Score of identification in range 2–3 indicates high-confidence identification, range 1.7–1.99 indicates low-confidence identification, and range 0.0–1.69 indicates no organism identification.

2.4. Screening of the bacterial antifungal activity on toxigenic fungal strains

A co-cultured method was performed to evaluate the antifungal activity of the bacterial strains. The bacterial strains were point inoculated in the center of the nutrient agar and incubated for 48 h. Spore suspensions in soft PDB (2.8 % potato dextrose) of the fungal strains (10^6 spore/ml) were overlayed around the bacterial colony as described by Rouse et al. (2008). The plates were incubated for 72 h at 28 °C. The mean of the inhibition zones was estimated from the readings recorded (n = 3).

2.5. Preparation of Bacillus species cell-free supernatant (CFS)

A single colony from 24 h culture in nutrient agar was transferred to 50 ml nutrient broth in 250 ml flask and was incubated in the shaker for 24 h at 37 °C and 200 rpm. The preculture of optical density (O.D) 0.1 was used to transfer 100 µl to 50 ml nutrient broth flasks and they were incubated in the same conditions for 48 h. The culture was centrifuged at 5000 rpm for 10 min and the supernatant was collected and stored at −20 °C for further use.

2.6. Investigation of the bacterial metabolites effect on the fungal growth

The CFSs were incorporated at different percentages (1–30 %) in a test tube of 50 ml PDB culture of *A. niger*, *A. flavus* and *Penicillium* (3 µl of 10^4/ml). The broth was amended with chloramphenicol (100 µg/l) and the flasks were incubated for 5 days. After that, the fungal broth cultures were filtered with nitrocellulose filter paper, and the biomass of mycelia was dried at 25 °C and measured in the treatment and control (Zeidan et al., 2019). The biomass mean was estimated from triplicates of different tubes for the control and treatment.

2.7. Investigation of the bacterial metabolites effect on the mycotoxins' synthesis

To investigate the effect of the CFSs on the fungal synthesize of mycotoxins, the fungal culture filtrate from the experiment in part ↱2.6 was obtained and used in ELISA to detect the mycotoxins concentration (Zeidan et al., 2019).

2.7.1. Effect of CSF on AFs synthesis

AFs Total ELISA kit manual used was RIDASCREEN® Aflatoxins total R4701, R-Biopharm AG, Darmstadt, Germany. 50 µl from the CFS and standards were added to the ELISA 96 wells, followed by 50 µl of conjugate. 50 µl of antibodies were added and the plate was mixed softly, covered, and incubated at 26 °C for 30 min. The liquid was poured out and the plate was cleaned on a dry tissue. The wells were filled with 250 µl distilled directly and were tapped on a tissue. The previous step was repeated twice. The substrate was added to the wells and the tray was incubated and covered for 15 min at 26 °C. 100 µl of the stop reagent were loaded and the absorbances were read within 30 afterwards.

2.7.2. Effect of CSF on OTA synthesis

OTA ELISA kit manual used was RIDASCREEN® Ochratoxin A 30/15, R-Biopharm AG, Darmstadt, Germany. 50 µl of CFS and standards were loaded in the wells followed by 50 µl of the diluted enzymes conjugate (1:11) and the tray was mixed gently and incubated in the dark at 26 °C for 30 min. The liquid was poured out and 250 µl of washing buffer were added. The wells were emptied, and the process was repeated twice. 100 µl of chromogen were added and the tray was mixed gently and incubated at 26 °C for 15 min. 100 µl of stop solution were added to the wells and the absorbance was read within 30 min afterwards.

2.7.3. Data acquisition

The absorbances of the mycotoxins were measured at 450 nm using a microplate reader (Tecan Sunrise™, Männedrof, Switzerland). The data were collected using Tecan-Magellan software, and the concentrations of mycotoxins were determined from a calibration curve containing six concentrations of each mycotoxin type, using RIDA®Soft Win-Z9996 software (R-Biopharm, Germany). The maximum limit of detection for AFs and OTA is >1800 ng/l and the minimum limit of detection is <50 ng/l.

2.8. Exploration of the bacterial metabolites impact on the mycelial morphology

The mycelia which were subjected to the treatment with different concentrations of the CFS of *B. amyloliquefaciens* obtained in section ↱2.6 were prepared to be visualized with the scanning electron microscope (SEM). The mycelia were filtered with normal filter paper and washed twice with twice with PBS solution (7.4 pH) by centrifuging at 2000 rpm/5 min. A small proportion of the mycelial pellet was obtained with a needle and fixed with 2.5 % glutaraldehyde for 2 h in clean 1.5 ml tubes. The pellet was dried with a gradient of ethanol solution (25 %, 50 %, 80 %, 100 %) each for 30 min. The dried pellet was mounted on silver holders and sputter sprayed with gold prior to visualization with SEM. Visualization were made possible by Nova NanoSEM 450 (Lv et al., 2011; Moghayedi et al., 2017).

2.9. Kinetics of the production of the antifungal activity

To study the kinetics of the antifungal metabolites production by the bacterial strains, cultures of nutrient broth were inoculated

with 100 µl inoculum from a pre-culture of OD 0.1. The subculture flasks were kept in the shaker at 37 °C/200 rpm for 48 h, and the CFSs were collected at different time intervals during the 48 h to explore their antifungal activity. To test the antifungal activities of CSFs, well-diffusion method was employed. In this method, a spore suspension (10^6 spores/ml) of *A. niger* was prepared in sterile distilled water amended with 0.05 % tween. In each PDA plate, 100 µl of fungal spore suspension was spread before drilling wells. In each well 100 µl of CFS were loaded. The plates were incubated for 72 h and the means of the inhibition zones were estimated from replicates (n=3) (Doshi et al., 2024).

2.10. Thermostability of the bacterial metabolites

To explore the thermostability of the CFSs, the CFSs were obtained from a 48 h bacterial broth by centrifuging at 5500 rpm/15 min. The CFSs were treated for 20 min with the following temperatures: −20 °C, 4 °C, 25 °C, 35 °C, 60 °C, 80 °C and 100 °C. A continuous treatment was obtained by subjecting the extract to all treatments together for 20 min. The effectiveness of the CFSs were tested by well-diffusion method in PDA against *A. niger* (↱2.9) (Zeidan et al., 2019).

2.11. Storage impact on the antifungal activity of the bacterial metabolites

The effect of storage on the bacterial CFSs was tested by storing the extracts at −20 ° C for different periods of time (Tenea et al., 2020). The extract of *B. subtilis* was experimented on day 7, 13 and 34 of storage, while the extract of *B. amyloliquefaciens* was experimented on day 7, 13, 34, 209, 215 and 230 of storage. The antifungal efficiency of the extract was examined after storage by well diffusion method (↱2.9).

2.12. Statistical analysis

One-way ANOVA (Analysis of variance) was performed to conduct the significance between the means of the treatment groups at $P = 0.05$. Tukey test was conducted for pairwise analysis of the groups means. Dunnett test was conducted to compare the significance of the groups means with the control means. Minitab software was used to conduct the mentioned tests.

3. Results

3.1. Identification of the bacterial strains exhibiting antifungal activity

In this study, bacterial strains isolated from the camel feed samples were tested for their antifungal activities. Two strains displaying promising antagonistic activities were subjected to identification by MALDI-TOF MS. The database matching based on the protein profile spectra of the two strains showed strong similarity with *B. subtilis* (score 1.71) and *B. amyloliquefaciens* (score 1.89). The strain identified as *B. subtilis* was isolated from the mixed-feed samples while *B. amyloliquefaciens* from the wheat bran. Gram staining of both strains showed Gram positive *Bacilli* as shown in the Fig. 1 below.

3.2. Screening the antifungal spectrum of the bacterial strains

To screen for the antifungal activity, the bacterial strains were co-cultured with the fungal spores. Toxigenic strains of *A. niger*,

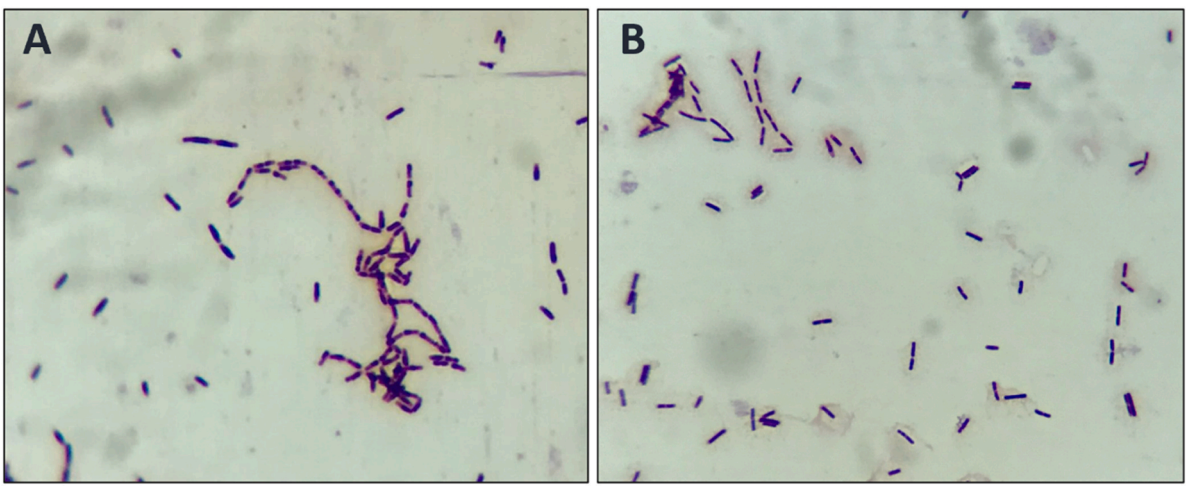

Fig. 1. Gram staining of bacterial strains with antifungal activity. “A” represents B. amyloliquefaciens and “B” represents B. subtilis (1000X).

Penicillium, and *Fusarium oxysporum* were used as antagonists to evaluate the antifungal activity of the bacterial strains. The diffusible metabolites of *B. subtilis* and *B. amyloliquefaciens* were shown to have a strong antifungal activity against the toxigenic fungal strains when incubated together (Fig. 2). The inhibition zones were observed for *B. amyloliquefaciens* against all fungal strains and *F. oxysporum* had lower inhibition zones (21.75 mm) compared to the other fungi. Strain *B. subtilis* exhibited a strong antifungal activity against all species except against *Penicillium* (Sp21).

3.3. Effect of bacterial CFSs on fungal growth and mycotoxins synthesis

3.3.1. Effect of bacterial CFSs on fungal growth

To explore the effect of *B. subtilis* and *B. amyloliquefaciens* CFSs on fungal growth, the CFSs were incorporated with the spores' cultures of *A. flavus, A. niger* and *Penicillium* at different concentrations (Fig. 3). The inhibitory activity of the bacterial extracts was explored against *A. flavus* starting from 5 % to 30 % (Fig. 3A). A gradual reduction in *A. flavus* biomass was observed when treated with both *Bacilli* CFSs. A significant reduction in biomass was obtained for *A. flavus* starting at 10 % treatment with both extracts. *Penicillium* was overly sensitive to the treatment with *B. amyloliquefaciens* CFS compared to that of *B. subtilis* (Fig. 3B). The biomass of *Penicillium* was significantly reduced when treated with 1 % of *B. amyloliquefaciens* CFS, and a complete inhibition of its biomass was obtained at 15 % treatment. The range of treatment with *B. subtilis* CFS started from 5 % to 30 % and a significant reduction in the fungal biomass was achieved at 5 %, however, no complete inhibition was accomplished for this fungus. *A. niger* was more sensitive upon treatment with *B. amyloliquefaciens* CFS as it was significantly reduced starting from 4 % and its biomass was completely inhibited at 10 % (Fig. 3C). The CFS of *B. subtilis* significantly reduced the growth of *A. niger* at 1 % and completely inhibited its growth at 30 %.

3.3.2. Effect of bacterial CFSs on mycotoxins synthesis

Among the characteristics of biocontrol agents is assessing their ability to inhibit the mycotoxins synthesis. Hence, after incorporating the CFS of *B. subtilis* and *B. amyloliquefaciens* in the fungal cultures, their effect on mycotoxins synthesis was estimated. The mycotoxins explored were OTA from *Penicillium* and *A. niger*, and AFs from *A. flavus* (Fig. 4). The incorporation of the *B. amyloliquefaciens* CFS with *Penicillium* culture resulted in a significant inhibition in OTA synthesis (Fig. 4A). At 2 %, the CFS has reduced OTA significantly (486.7 ng/l) compared to the control which had a concentration of OTA above ELISA detection limit (>1800 ng/l). The CFS of *B. subtilis* had shown a significant reduction in OTA synthesis from *Penicillium* starting at 5 % (68.4 ng/l) compared to the control (>1800 ng/l) (Fig. 4B). The CFSs of both bacteria were explored against *A. niger* strain, and it was observed that CFS of *B. amyloliquefaciens* had significantly reduced OTA at 10 % (193.8 ng/l) compared to the control (>1800 ng/l) (Fig. 4C). Further treatments with *B. amyloliquefaciens* CFS resulted in the reduction of OTA concentrations below ELISA detection limit (<50 ng/l). A significant reduction of OTA from *A. niger* by *B. subtilis* CFS was accomplished at 20 % (144.4 ng/l). The treatment with both CFSs against *A. flavus* had resulted in significant reduction at 5 %, at which the concentrations of AFs were 212.4 ng/l and 365.5 ng/l by *B. amyloliquefaciens* and *B. subtilis* CFSs, respectively. The concentration of AFs reached an average of 1770.5 ng/l in the control of *A. flavus*.

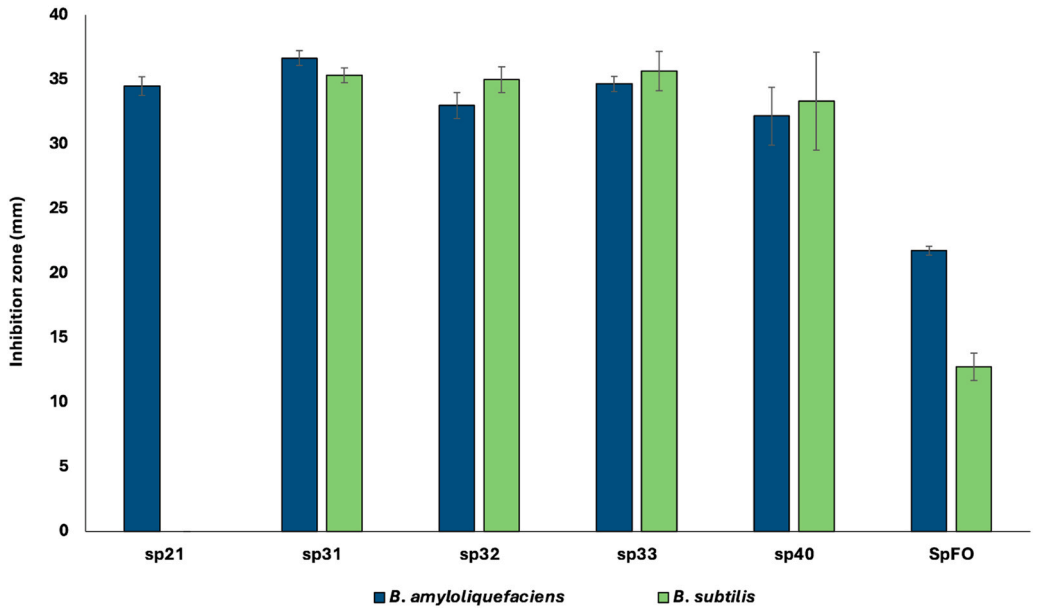

Fig. 2. Screening of the antifungal activity of *B. subtilis* and *B. amyloliquefaciens* against toxigenic fungal strains in overlaying assay method. Sp21: *Penicillium*; Sp31–33, Sp40: *A. niger*, SpFO: *Fusarium oxysporum*.

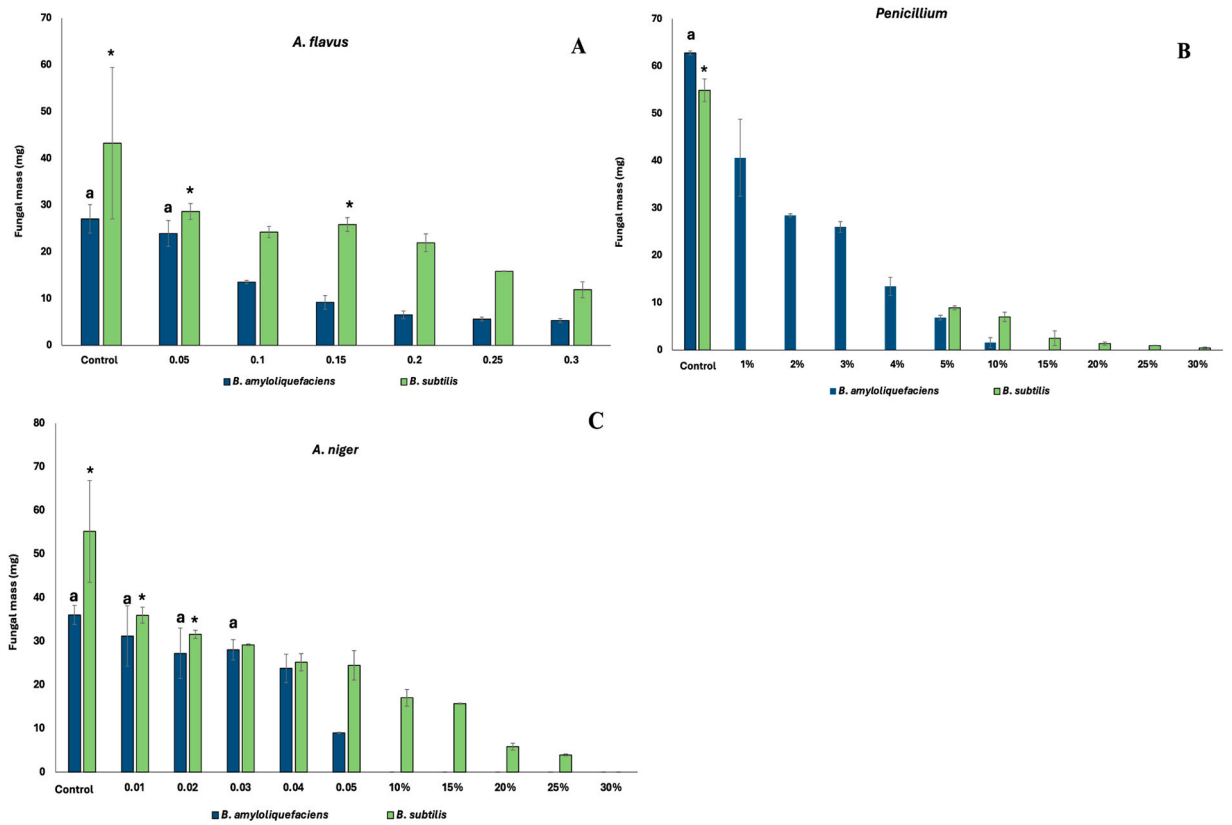

Fig. 3. Inhibition of the average mycelial growth (mg ±SD) of *A. flavus* (A), *Penicillium* (B), and *A. niger* (C) upon treatment with CFSs of B. subtilis and B. amyloliquefaciens at different percentages after 5 days incubation. Dunnett test was used to compare the significance of the treatments with the control group for *B. amyloliquefaciens* at $p \leq 0.05$ (a) and for *B. subtilis* at $p \leq 0.05$ (*). Mean bars not labeled with the letter or asterisk are significantly different from the control's mean level.

3.4. Effect of bacterial CFSs on the mycelial morphology

In this study, the impact of *B. amyloliquefaciens* CFS was explored against *A. niger* and *Penicillium* at different concentrations in SEM imaging (Fig. 5). *A. niger* was exposed to 1–5 % CFS and *Penicillium* was exposed to 1–10 %. The increase in CFS concentration had a clear inverse effect on the biomass of both fungal strains. More inhibition was observed when the percentage of CFS was increased. The CFS of *B. amyloliquefaciens* caused distinguishable morphological changes in both *A. niger* and *Penicillium* hyphae. In the control, the morphology of the hyphal surface was smooth, and their cylindrical shape was maintained. In contrast, mycelia treated with CFS exhibited a different pattern of changes, including a rough surface appearance and a distorted cylindrical shape. The increased exposure of the hyphae to the antifungal CFS caused them to become more flattened, twisted and wrinkled. This observation was consistent in both fungal strains. The presence of minute vesicles can be discernible on the surface of the treated hyphae.

3.5. Kinetics of the bacterial antifungal metabolites

The production of the antifungal compounds by *B. amyloliquefaciens* and *B. subtilis* was assessed at different growth stages for 48 h. The CFSs were collected, and their antifungal activity was tested against *A. niger* in well-diffusion method (Fig. 6). The antifungal activity of *B. amyloliquefaciens* CFS began to show after 24 h of incubation, with a mean inhibition zone of 20.2 mm (Fig. 6). The antifungal activity of *B. amyloliquefaciens* CFS continued to increase proportionally with the incubation time and the maximum activity was reached at 48 h (21.3 mm). Likewise, for *B. subtilis* CFS, its activity began to appear at 12 h showing a mean inhibition zone of 13.5 mm (Fig. 6). The CFSs activity continued to increase, reaching maximum inhibition at 27 h, with an inhibition zone of 19.8 mm.

3.6. Thermostability of the bacterial antifungal metabolites

The antifungal metabolites have been explored for their heat stability by exposing the CFS to various temperatures for 20 min. The antifungal activity of the heat-treated CFSs was evaluated in well-diffusion method against *A. niger*. The maintenance of antifungal activity in the CFS of *B. amyloliquefaciens* and *B. subtilis* after treatments indicates their high stability at all tested temperatures,

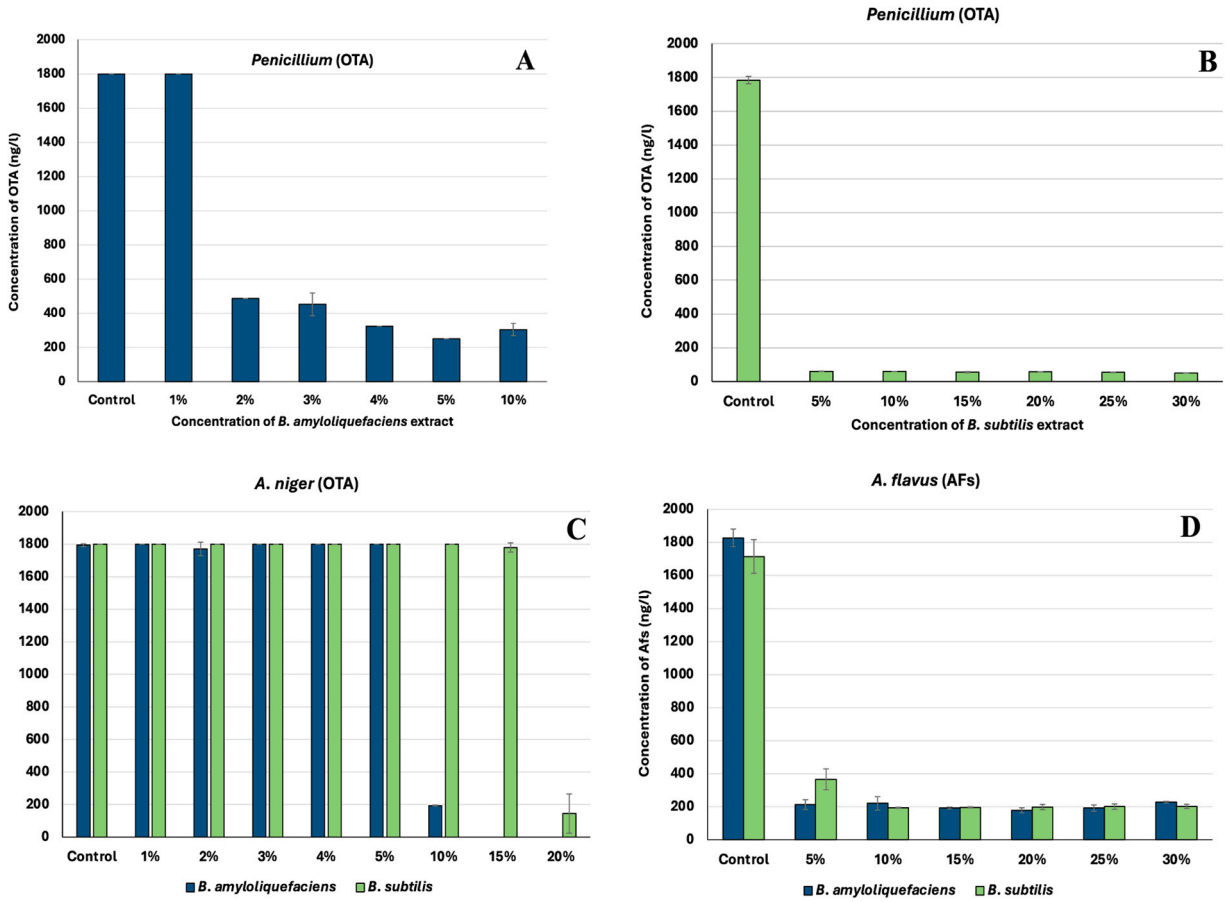

Fig. 4. The impact of B. amyloliquefaciens and *B. subtilis* CFSs on the inhibition of mycotoxin synthesis from strains *Penicillium*, *A. niger* and *A. flavus* (ng/l ± SD). OTA levels were reported for *Penicillium* (A & B) and *A. niger* (C), and AFs total levels were reported for *A. flavus* (D) at different percentages, all on the 5th day of incubation. Dunnett test was used to compare the significance of the treatments with the control group at $p \leq 0.05$. Means not labeled with the letter A are significantly different from the control's mean level.

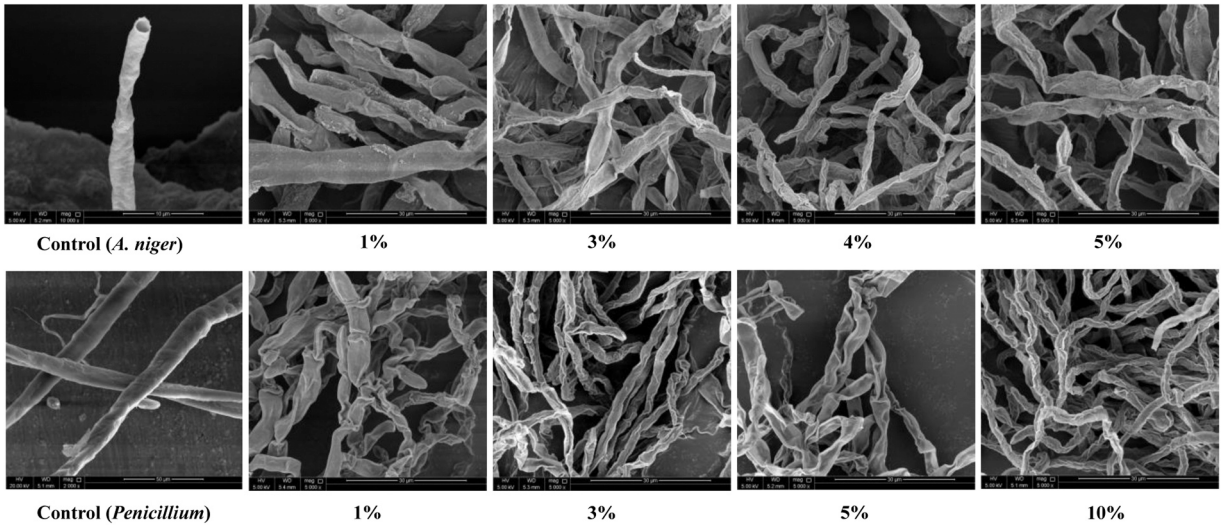

Fig. 5. Scanning electron microscope (SEM) observations for the effect of B. amyloliquefaciens antifungal CFS on the morphology of *A. niger* and *Penicillium* hyphae. The untreated hyphae are labeled as "control", and the treated hyphae are labeled with the CFS concentrations (%). A. niger was exposed to 1 %, 3 %, 4 %, 5 % of CFS and Penicillium was exposed to 1 %, 3 %, 5 %, 10 %.

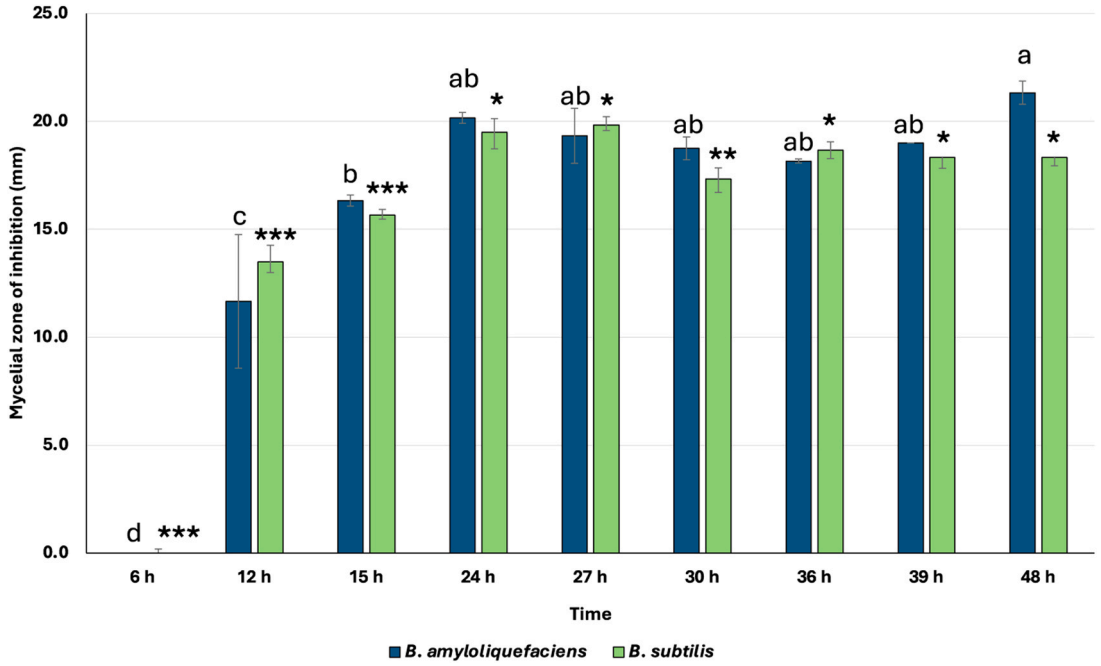

Fig. 6. The kinetics of antifungal activity of *B. amyloliquefaciens* and *B. subtilis* CFS against *A. niger* in well-diffusion method during 48 h culture. The mycelial inhibition zones were reported (mm± SD) for each reading after 48 h. Tukey test was conducted to estimate significantly different means of the treatment groups for *B. amyloliquefaciens* at $p \leq 0.05$ (a), $p \leq 0.01$ (b), $p \leq 0.001$ (c) and $p \leq 0.0001$ (d) and for *B. subtilis* at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). Mean bars that do not share a letter or asterisk are significantly different from each other.

including 100° C. The inhibition zones of *A. niger* detected by *B. amyloliquefaciens* CFS were shown to be insignificant from the zones obtained for the control (37° C) (Fig. 7). Both CFSs were treated with all temperatures at once to test their durability to heat treatments, and it was shown that they still retained a strong antifungal activity.

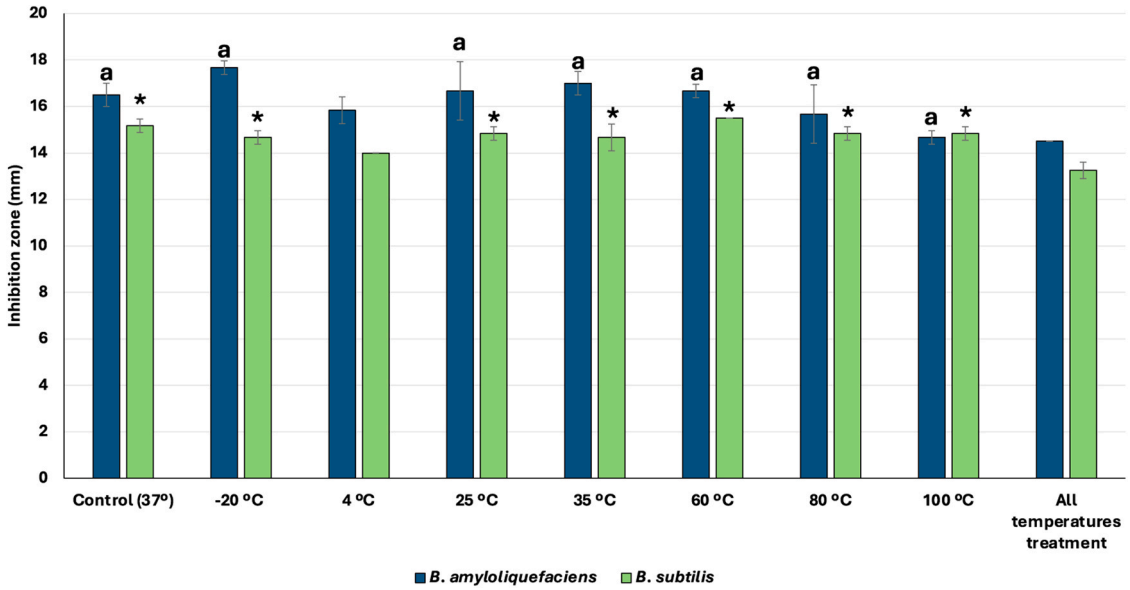

Fig. 7. Heat stability of the antifungal CFS of *B. amyloliquefaciens* and *B. subtilis* explored against *A. niger* in well-diffusion method. Both CFSs were treated with the following temperatures: −20 °C, 4 °C, 25 °C, 35 °C, 60 °C, 100 °C and then all temperatures at once. Dunnett test was used to compare the significance of the temperature's treatments with the control group for *B. amyloliquefaciens* at $p \leq 0.05$ and for *B. subtilis* at $p \leq 0.05$. Mean bars not labeled with the letter or asterisk are significantly different from the control's mean level.

3.7. Effect of storage on bacterial antifungal metabolites

The effect of storage period on *B. amyloliquefaciens* and *B. subtilis* CFSs was investigated. The CFS of *B. amyloliquefaciens* retained its antifungal activity throughout the storage period (Fig. 8). The inhibition zone obtained for this CFS on the 7th day was not significantly different from the zones obtained on the other storage days. Storing *B. amyloliquefaciens* CFS up to 236 days didn't affect its activity. The CFS of *B. subtilis* was stored up to 34 days only, and it continued to exhibit a strong antifungal activity. It was observed that both CFSs retained their antifungal activity at all periods of storage.

4. Discussion

Bacillus species are widely recognized and explored for their antifungal activity against crops or plants pathogens (Cruz-Martín et al., 2023; Tuyen et al., 2023). Two *Bacillus* strains were isolated from mixed feed and wheat bran. Their MALDI-TOF identification revealed that they are *B. amyloliquefaciens* and *B. subtilis,* respectively.

When explored for their antifungal activity, both strains exhibited strong antifungal effects and inhibited toxigenic fungal strains from various genera. The varied antifungal activity performed by *B. amyloliquefaciens* and *B. subtilis* against strains from *A. niger*, *A. flavus, Penicillium* and *F. oxysporum* confirms that their antifungal activity is strain selective. This conclusion was drawn by Rosier et al. (2023) and Kaur et al. (2023), who purified and studied the activity of antifungal compounds from *Bacillus* spp. They reported that these antifungal metabolites tend to exhibit strain-specific antagonistic activity.

The CFS of the isolated *Bacillus* strains has been examined for its effectiveness in inhibiting fungal growth in liquid cultures. The CFS of *B. amyloliquefaciens* showed the highest inhibitory effect against *A. niger,* while *A. flavus* was the least sensitive. Conversely, *B. subtilis* was most effective against *A. niger* and least effective against *A. flavus*. Applying the highest concentration of the CFSs in the fungal culture was proportionally correlated to the inhibition of the fungal biomass. This indicates that the growth inhibitory process is dose-dependent. *B. amyloliquefaciens* had been previously reported to inhibit the fungal biomass of *Aspergillus* and *Penicillium* species (Ayed et al., 2015; Kadaikunnan et al., 2015). Kadaikunnan et al. (2015) prepared cellular extracts from *B. amyloliquefaciens* that exerted an antagonistic activity against all tested fungal groups, including *A. niger* and two *Penicillium* strains. Comparatively, it can be deduced that *B. amyloliquefaciens* possesses a slightly stronger antagonistic activity than *B. subtilis.* This observation is consistent with the findings of Siahmoshteh et al. (2017), who reported that *B. amyloliquefaciens* exhibited slightly stronger activity against the radial growth of *A. parasiticus* compared to *B. subtills.*

The impact of *B. amyloliquefaciens* and *B. subtilis* CFS on reducing mycotoxins was evaluated. Both strains were observed to reduce mycotoxins synthesis by the fungal strains after the addition of the CFS. Even at low concentrations, such as 2 % or 5 %, a significant reduction in OTA and AFs production by *Penicillium* and *A. flavus*, respectively, was observed. Therefore, it can be said that the reduction in mycotoxins synthesis was partially dose dependent. There was a significant reduction in *A. niger* or *Penicillium* biomass and their correspondent mycotoxins. However, this trend did not apply to all strains, as seen in the case of *A. flavus*. For example, increasing the concentration of *B. subtills* CFS up to 15 % reduced the fungal biomass but not significantly compared to the control. This suggests that while fungal biomass may not be significantly inhibited or reduced, the synthesis of their toxins can still be affected. This observation was consistent when using both bacterial CFSs. It was noted that the CFS of *B. amyloliquefaciens* was slightly more effective in reducing the synthesis of AFs from *A. flavus* compared to *B. subtilis* CFS. *B. amyloliquefaciens* was observed to be more efficient in inhibiting OTA synthesis from *Penicillium* and *A. niger*. Similarly, a great reduction in AFs from *A. flavus* was also observed. This conclusion is supported by Siahmoshteh et al. (2017), they found that inoculating pistachio kernels with *B. amyloliquefaciens* and *B. subtilis* and then adding *A. flavus* showed that *B. amyloliquefaciens* (54.9 %) performed better than *B. subtilis* (45.9 %) in removing AFs from the kernels.

Bacteria from the *Bacillus* genus are known to inhibit the growth of toxigenic fungi and their associated mycotoxins. *B. subtilis* strains are particularly effective inhibitors of AFs and OTA (Afsharmanesh et al., 2018; Shukla et al., 2018). Nievierowski et al. (2023) studied the effect of the biological control on grapes must contaminated with *A. carbonarius* after treatment with *B. velezensis*. They found that OTA and its six precursors were not detected in the samples treated with *B. velezensis*.

The effect of the bacterial antifungal supernatant of *B. amyloliquefaciens* and *B. subtilis* on the morphology of fungal hyphae was evaluated using SEM. SEM revealed a pronounced effect of the antifungal metabolites on the morphology of fungal mycelia. Concordant to the growth and toxins reduction, there was a proportional increase in damage to the hyphal structure with increasing concentration of the CFSs. Higher percentages of CFSs led to more wrinkling and flattening of the cell wall. Liu et al. (2023) demonstrated that when they treated *F. incarnatum* with *B. amyloliquefaciens* lipopeptide, changes in the morphology were observed including swellings and cytoplasmic aggregations in hyphae.

To study the kinetics of antifungal agents produced by *B. amyloliquefaciens* and *B. subtilis*, their CFSs were tested against *A. niger* at different stages over 48 h. Our data indicate that the production of the antifungal metabolites by both *Bacillus* strains is associated with their growth stage. In this case, the antagonistic effect of the bacterial CFSs is attributed to the production of antifungal metabolites in the broth culture. Fig. 6 shows the four phases of the growth curve (lag: 0–6 h, log: 6–24 h, stationary: 24–48 h). The highest levels of inhibition were observed later, after the exponential phase, indicating that the antifungal metabolites of *B. amyloliquefaciens* and *B. subtilis* are secondary metabolites produced at different stages and in varying amounts. This conclusion is supported by Xu et al. (2019), who found that the antagonism of *B. amyloliquefaciens* against *Fusarium oxysporum* f. sp. *niveum* was more effective (inhibition percentages ranging from 4.50 % to 61.06 %) when the bacterial CFS was collected between 12 and 72 hour. In parallel with our results, Hanene (2012) demonstrated that the best activity of *B. subtilis* SR146 antifungal metabolites against *Fusarium* strains and *Botrytis* occurred between 24 and 48 hours.

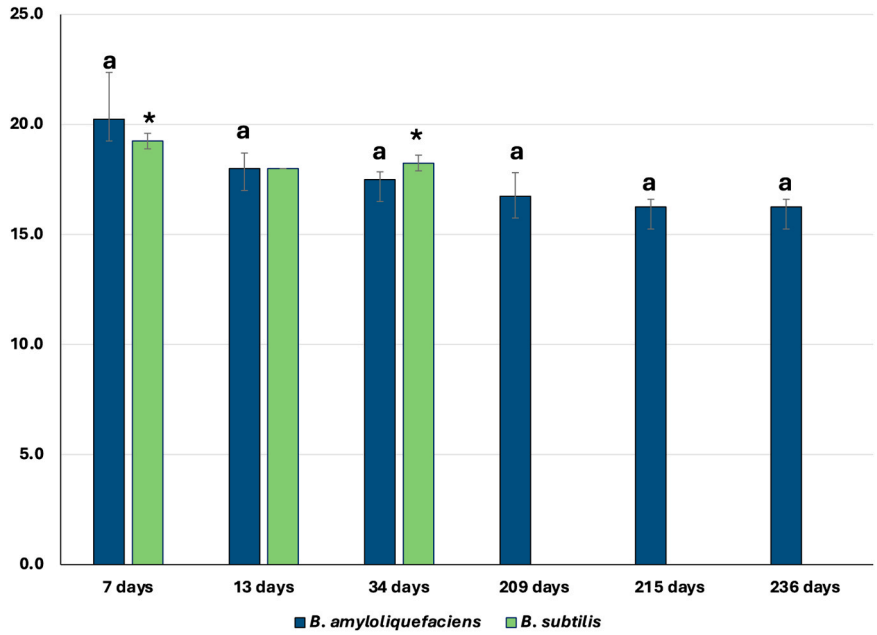

Fig. 8. Stability of the antifungal activity of *B. amyloliquefaciens* and *B. subtilis* CFSs upon various storage periods tested against *A. niger* in well-diffusion method. Tukey test was conducted to estimate significantly different means of the treatment groups for *B. amyloliquefaciens* at $p \leq 0.05$ and for *B. subtilis* at $p \leq 0.05$. Mean bars not labeled with the letter or asterisk are significantly different from the others.

The thermal resistance of the bacterial antifungal CFSs was evaluated and it was observed that the thermally treated CFSs retained their antagonism against *A. niger*. The significance of the CFSs obtained from *Bacillus* strains lies in their ability to be used in various applications due to their high stability even at 100° C. The thermal stability of the antifungal metabolites is thought to be linked to their classification as polypeptides, lipopeptides or proteins (Li et al., 2021). Additionally, the presence of the antifungal metabolites in a mixture contributes to their stability (Lin et al., 2020). Tuyen et al. (2023) reported that a purified *B. subtilis* extract with antifungal activity remained stable and retained its antifungal activity at 100 ° C when heated for 15 min. Kilani-Feki et al. (2016) studied the activity of *B. subtilis* V26 CFs and found it to be thermally stable up to 70 °C.

One common challenge of developing fungicides based on natural compounds or biological extracts is their stability and storability (Matrose et al., 2021). This research work provides evidence of the stability of the bacterial CFSs over long periods of storage. The CFSs of *B. amyloliquefaciens* and *B. subtilis* were stored in fridge for 236 and 34 days, respectively, and their antagonistic activity against *A. niger* was confirmed to be stable in long period storage.

5. Conclusion

Fungal infections pose significant challenges to food and feed commodities at both pre- and post-harvest stages. In this study, we isolated candidate *Bacillus* strains (*B. amyloliquefaciens* and *B. subtills*) exhibiting promising antifungal activity against mycotoxigenic fungi, and significantly reducing mycotoxins synthesis. The antifungal CFSs of both strains exhibited exceptional thermal stability and retaining their antagonistic activity after long-term storage. This thermal stability and storability make the bacterial CFSs valuable for potential applications, including field use and in the food industry where stability during processing is crucial. We recommend further identification and purification of the antifungal metabolites from the bacterial supernatants to decipher their mechanism of action.

CRediT authorship contribution statement

Roda Al-Thani: Validation, Formal analysis, Conceptualization. **Mohammad Yousaf Ashfaq:** Methodology. **Zahoor Ul Hassan:** Writing – review & editing, Validation, Methodology, Formal analysis, Conceptualization. **Samir Jaoua:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Randa Zeidan:** Writing – review & editing, Writing – original draft, Validation, Methodology, Conceptualization.

Declaration of Competing Interest

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

Data availability

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

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