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חקר מיקרוביום של גרעיני חיטה לאחר הקציר: פיתוח אמצעים להדברה ביולוגית

Analysis of stored wheat grain microbiome: development of a biocontrol strategy to promote the product quality, safety and health

מוגש לקרן המדען הראשי במשרד החקלאות ע"י

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תקציר

חיטה ומוצריה מהווים מרכיב מרכזי בתפריט המזון בכלל וכמקורות חשובים של אנרגיה וחלבון בפרט לבני אדם ובעלי חיים. נהוג לאחסן את החיטה לאחר הקציר לתקופה ממושכת ובשל כך התוצרת חשופה למזיקים ופטריות שונות הגורמות להפסדים רבים ומהווים סיכון בריאותי לאדם ובעלי החיים. פטריות עובש עלולות להתפתח על מגוון רחב של תוצרת חקלאית טרייה ויבשה מאוחסנת תוך כדי יצירת מטבוליטים שניוניים הרעילים ליונקים ומוגדרים כמיקוטוקסינים שעשויים לחדור לשרשרת המזון ולגרום לפגיעה באיכות ובטיחות מזון.

לקהילות מיקרוביאליות על משטחי הצמח יש השפעה על בקרת גורמי מחלות באמצעות האינטראקציה שלהן עם צמחים פונדקאים ופתוגנים, ומכאן חקר המיקרוביום פותח הזדמנויות מחקר חדשות שלא היו קיימות בעבר. פיתוח טכנולוגיות של ריצוף של הדור הבא (next generation sequencing) מאפשר גישות חדשות של חקר אוכלוסיות מיקרוביאליות ואינטראקציות ביניהן, הרבה מעבר לטכניקות מסורתיות. לכן, מטרת המחקר היא לחקור את מיקרוביום של גרעיני חיטה לאחר הקציר על מנת לפתח גישות חדשות להדברה ביולוגית של זיהומים הנגרמים ע"י פטריות עובש ורעלנים בגרעינים מאוסמים. במהלך שנה ראשונה של המחקר ביצענו אנליזת מיקרוביום של גרעיני חיטה שלמים לאחר הקציר בכדי לשלב מידע זה במציאת פתרונות הדברה ביולוגיים של עובשים מיקוטוקסיגניים בחיטה מאוחסנת. במהלך שנה שנייה ביצענו ריצוף עמוק של 16S rRNA ואזור ITS מ- DNA אפיפיטי ואנדופיטי של גרעיני חיטה על מנת להבין טוב יותר אוכלוסיות אפיפיטיות ואנדופיטיות של גרעיני חיטה. בנוסף, בודדנו מיקרואורגניזמים האפיפיטיים והאנדופיטיים מגרעיני חיטה על מצעי מזון שונים. לאחר סריקה של כל התבדידים לפעילות אנטיפטרייתית נמצאו ארבעה תבדידי חיידקים (בצילוס) וארבעה תבדידי שמרים בעלי פעילות כנגד פטריות מיקוטוקסיגניות. זני בצילוס היו המעכבים היעילים ביותר של צמיחת פטריות מיקוטוקסיגניות השכיחות בגרעיני חיטה, *Aspergillus flavus*, *Fusarium proliferatum* ו-*Alternaria infectoria*. בשנה שלישית של המחקר, יישמנו פרוטוקול לגידול בהיקף רחב (חצי מסחרי) של זני בצילוס בעלי פעילות אנטיפטרייתית, ונבדקה יעילותם כנגד פטריות מיקוטוקסיגניות בגרעיני חיטה מאולחים. כמו-כן, פטריית

שמר *Rhodotorula glutinis* שבודדה מגרעיני חיטה, הראתה יכולת לפרק מיקוטוקסינים הן במצע גידול (*in vitro*) והן בגרעיני חיטה מאולחים (*in vivo*). הבנה טובה יותר של יחסי הגומלין המורכבים בתוך הקהילות המיקרוביאליות של גרעיני חיטה מאוחסנים תסייע בפיתוח אסטרטגיות של הדברה ביולוגית על מנת להתגבר על זיהומי פטריות מיקוטוקסיגניות ומיקוטוקסינים בדגניים.

רשימת פרסומים והרצאות שנבעו מהמחקר:

1. Solanki, M.K., Abdelfattah, A., Britzi, M., Zakin, V., Wisniewski, M., Droby, S. and Sionov, E. (2019). Shifts in the composition of the microbiota of stored wheat grains in response to fumigation. *Front. Microbiol*. 10:1098. https://doi.org/10.3389/fmicb.2019.01098
2. Sionov E. (2019). Exploring wheat grain microbiome during storage and its use for biocontrol of mycotoxigenic fungi. U.S. – Israel Binational Agricultural Research and Development (BARD) conference, Leesburg, VA, USA. (Lecture).
3. Solanki, M.K., Abdelfattah, A., Zakin, V., Wisniewski, M., Droby, S. and Sionov, E. (2021). Analysis of stored wheat grain associated microbiota reveals biocontrol activity among microorganisms against mycotoxigenic fungi. In preparation.

Contents

Introduction

Wheat is one of the essential cultivated grain crops and is an important source of calories and plant-derived protein in human food. The maintanence of high-quality and healthy wheat grains is essential for the stability of the world's food supply and for global food security. Wheat grains, like seeds of other cereal crops, are colonized by complex of epiphytic and endophytic microbial communities that play an imporant role in grain health, quality and susceptibility to disease (Links et al., 2014). Microorganisms, both bacteria and fungi, naturally occurring within cereal crops without causing any damage and may influence host growth and development. Conversely, some specific crop seed-associated microbes can cause spoilage, decreasing crop value, and may have a harmful effect on human health. When grains are colonized by moulds there is a significant risk of contamination with mycotoxins, which are toxic chemical products, formed as secondary metabolites by mycotoxigenic fungi. Many species of *Fusarium*, *Aspergillus*, *Penicillium*, and *Alternaria* are not only recognized as plant pathogens but are also sources of important mycotoxins of concern in relation to animal and human health (Placinta et al., 1999; Magan et al., 2010). Poor postharvest management can lead to rapid deterioration in quality, with severe decreases in germinability and nutritional value of stored grain, possibly accompanied by undesirable fungal contamination and, consequently, toxin production (Magan et al., 2003). Mycotoxigenic fungal species may cause significant wheat grain yield losses in the field and storage facilities, especially due to their ability to produce mycotoxins, making crops unsafe for consumption (Chen et al., 2019; Ferrigo et al., 2016; Lee and Ryu, 2017; McCormick et al., 2011; Streit et al., 2013). Consistently effective control measures against mycotoxigenic fungi are lacking. Current disease management practices are primarily based on the use of synthetic fungicides, which have been proven to be effective against several genera of mycotoxigenic fungi. However, the increasing awareness of the harmful side-effects of these chemical compounds on environmental and human health forces us to find efficient, less toxic alternative strategies to control fungal infection. Biological control using microbial antagonists against fungal plant pathogens offers an alternative approach to be used as part of an integrated management strategies for prevention and control of mycotoxigenic fungi and mycotoxins in stored wheat. The plant protective ability of some bacterial epiphytes and endophytes against fungal pathogens has been reported in several crops, including wheat (Gdanetz and Trail, 2017; Links et al., 2014; Mousa et al., 2016; Comby et al., 2017; Díaz Herrera et al., 2016).

Nevertheless, one of the major drawbacks associated with use of biological control agents is their inconsistent efficacy over time (Baffoni et al., 2015; Musyimi et al., 2012). Most commercially available biocontrol isolates do not originate from the plants they are intended to protect, which might at least partially explain their inconsistent efficacy in the field and/or storage warehouses. Exploring the diversity of stored wheat grain microbiome might be helpful in identifying new microbes with potential antifungal and antitoxigenic activity. Microorganisms naturally occurring within wheat seeds without causing any damage to their host can be good candidates as biocontrol agents. For this reason, the major goal of the present study was to classify and characterize epiphytic and endophytic microbiomes of stored wheat grains. Moreover, we used the wheat microbiome analysis in the context for identifying of microorganisms with potential antagonistic properties, isolated from stored seeds, for the biological control of important disease-causing mycotoxigenic fungi and their toxins.

The research goals of the study are (as specified in the proposal):

1. To analyze the stored wheat grain microbiome using high-throughput (next generation) sequencing of the bacterial 16S rRNA gene and fungal internal transcribed spacer (ITS) region
2. Analysis of epiphytic and endophytic microflora of stored wheat grains and determination of the level of compatibility between the composition of the microbial communities observed by bioinformatics analysis of microbiome and the group of isolated strains
3. Examination of potential isolates for biological control - testing microorganisms with antagonistic activity against a wide range of mycotoxigenic fungi (such as *Aspergillus, Fusarium, Alternaria*)
4. Examination of the ability of microorganisms with the potential of biolcontrol activity to biodegrade mycotoxins (such as aflatoxins, ochratoxin A, fumonisins)
5. Studying the mechanism of action of bacteria and/or yeast isolates found to be effective in inhibiting mycotoxigenic fungi and decomposition of mycotoxins on wheat grains

Materials and methods

Wheat grain samples. Wheat grain (*Triticum aestivum*) samples were collected from seven wheat grain storage facilities located in northern and southern districts of Israel. In each storage facility, three samples (1 kg of grain each), destined for human consumption, were collected (3 samples × 7 storage sites = a total of 21 samples) from

the front face and the center, at points located 1 m in horizontal depth within the grain mass, and from areas close to the walls. Grain temperature and moisture content were in the ranges of 27–33 °C and 10.5–12.9%, respectively. The collected samples were kept in sterile plastic bags during transport to the laboratory.

DNA Extraction. To isolate epiphytic DNA, a 10 g of each sample was soaked in a solution of 45 ml buffered peptone water (10 g peptone, 5 g NaCl, 3.5 g Na_2HPO_4, 1.5 g KH_2PO_4 l^{-1} containing 0.05% Triton X-100 (Sigma) in a 250 ml Erlenmeyer flask at room temperature with shaking (150 rpm) for 1 h. The liquid fractions were centrifuged at 4000 g for 15 min and the supernatant discarded. Pellets were frozen in liquid nitrogen, and freeze-dried pellets were used to extract the DNA. Endophytic DNA was isolated after surface sterilization of wheat grains with 3% active sodium hypochlorite (NaOCl) for 2 min, 70% ethanol for 2 minutes, and rinsed five times in sterile distilled water. Surface sterilized wheat grains (10 g) were frozen in liquid nitrogen, freeze-dried and milled into a fine powder with a grain grinder. The grain grinder was cleaned and disinfected with 70% ethanol solution between sample grinding and powder was used for DNA isolation. Epiphytic and endophytic samples DNA extraction was performed as previously described (Sadhasivam et al., 2017).

Gene amplification and sequencing. 16S rDNA gene for bacteria and the ITS rRNA gene for fungi were amplified and sequenced in order to describe the stored wheat grain samples microbial community. PCR amplification of each sample was performed in triplicate, PCR products were pooled and purified with PCR clean-up kit. Amplicons were sequenced at the University of Illinois at Chicago, Center for Genomic Research using a dual-index barcode strategy and Illumina MiSeq 2 x 300 bp chemistry.

Bioinformatics. The QIIME pipeline was used for quality filtering, trimming, operational taxonomic unit (OTU) clustering with abundance data and associated taxonomic annotations, and chimera detection. The OTU table was normalized by rarefaction to an even sequencing depth in order to remove sample heterogeneity. The rarefied OTU table was used to calculate alpha diversity indices including Observed Species (Sobs), Chao1, and Shannon metrics. Alpha diversities were compared based on a two-sample *t*-test using nonparametric (Monte Carlo) methods and 999 Monte Carlo permutations. The cumulative sum scaling normalized OTU table was analyzed using the Bray Curtis metrics (Bray and Curtis, 1957) and utilized to evaluate the β-diversity and construct PCoA plots (Vázquez-Baeza et al., 2013). Differential OTU abundance of the most abundant taxa ($\geqslant$0.1%) between sample groups were determined using a

t-test and the Kruskal–Wallis test (Kruskal and Wallis, 1952). In all tests, significance was determined using 999 Monte Carlo permutations, and the false discovery rate (FDR) was used to adjust the calculated *P*-values and when the FDR $P<0.05$ it was considered significant. Cytoscape 3.3.0 (www.cytoscape.org) was used to analyze the most abundant taxa ($\geqslant$0.1%) and construct network figures visualizing the interactions between significantly different taxa ($P<0.01$).

Identification of representative taxa. Standard QIIME analyses only provide a reliable identification of fungi down to the genus level. Therefore, the identity of most sequences were manually re-checked using BLAST searches of GenBank and Fungal Barcoding Databases (http://www.fungalbarcoding.org/). Furthermore, sequences representative of relevant OTUs were phylogenetically analyzed along with closely related reference sequences to enable their identification with the highest level of accuracy possible.

Isolation of epiphytic and endophytic isolates from wheat grain. The wheat samples were analyzed for presence of bacterial and yeasts isolates, and potentially mycotoxigenic fungi. In order to isolate epiphytic microbes from wheat grains, a 10 g sample of seeds was incubated in 90 ml of sterile saline (0.9% NaCl) solution; for isolation of endophytic microorganisms – surface sterilized wheat grains (10 g) were milled with a grain grinder and incubated in 90 ml of saline. The samples were incubated with shaking (150 rpm) at 28°C for 1h, then 100 µL of serial 1/10 dilutions were plated on Luria-Bertani (LB) agar plates for isolation of bacteria and Potato Dextrose Agar (PDA) plates supplemented with chloramphenicol (20 mg/ml) for isolation of filamentous fungi and yeasts. LB agar plates were incubated at 37℃ for 24–48 h, whereas PDA plates were incubated at 28℃ for 48–72 h. Bacterial colonies were randomly selected from the plates and streaked on fresh culture media to obtain pure cultures. Filamentous fungi and yeasts were transferred singly to PDA plates and subcultured twice to obtain a pure culture.

DNA was extracted from each bacterial strain using a lysozyme lysis method described by De et al. (2010). Fungal DNA extraction was performed on lyophilized mycelium/yeasts cells using CTAB-based method as previously described (Sadhasivam et al., 2017). DNA quality and yield were determined using a NanoDrop One spectrophotometer. The 16S rRNA gene in bacteria and ITS rRNA gene region in fungi were amplified with universal primers 27F/1492R and ITS1/ITS4, respectively. PCR

products were purified and sequenced through standard Sanger sequencing; sequences were identified via BLAST matches to the NCBI database.

Quantitative real-time PCR. qPCR was performed to quantify the presence of mycotoxigenic fungi, such as *Fusarium*, *Aspergillus*, and *Alternaria* spp., in the stored grains. A broad-spectrum primer pair ITSPF/ITSPR was used to target the fungal ITS region and single genus-specific TaqMan probes were tested for their ability to detect fungal DNA. The probes were labeled on the 5' end with the fluorescent reporters FAM, HEX and TEX, and on the 3' end with TAMRA, BHQ1, and BHQ2. The qPCR reactions were carried out using qPCRBIO Probe Mix (PCR Biosystems, London, United Kingdom), 10 μM of each primer, 10 μM of each probe, and 5 ng of genomic DNA template. Amplification reactions were performed in a total reaction volume of 10 ml and were run on the Eco Real-Time PCR System (Illumina). Real-time qPCR reactions were performed in triplicate for each biological replicate and each sampling date contained three biological replicates. The cycle threshold value (Ct), which refers to the cycle number where the sample's fluorescence significantly increases above the background level, was calculated automatically by the Eco software. The detection limit of the probes was assessed under optimized PCR conditions with the DNA extracted from pure cultures of *Fusarium proliferatum* (NRRL 31866), *A. flavus* (NRRL 3518), and *Alternaria infectoria* (F11, isolated from stored wheat grains). Serial dilutions were made of DNA from the three fungal species. The real-time PCR data indicated that the Ct values correlated well with known DNA quantities from 8 pg to 5 ng. To test the efficacy of the method, sterilized wheat grain samples (10 g) were inoculated with each of *A. flavus*, *F. proliferatum*, and *A. infectoria* cultures at final concentration of 10^4 spores/g and incubated for 3 days. Non-inoculated sterilized grain samples used as control. DNA extracts isolated from the samples were analyzed using the qPCR assay. The obtained Ct values (23–30) revealed high reproducibility and demonstrated the ability to quantify fungal DNA in artificially contaminated wheat grains. qPCR analysis indicated the average values of DNA content for *F. proliferatum*, *A. flavus*, and *A. infectoria* spp. in seeds at concentrations of 268, 44 and 30 ng/mg, respectively.

Assays for antifungal (antagonistic) activity of bacterial and yeast isolates. *Dual Culture Test*

The antagonistic activity of the bacterial isolates against mycotoxigenic *Aspergillus*, *Fusarium,* and *Alternaria* was tested by dual culturing of the bacteria and fungi on PDA plates. On each agar plate, one bacteria pre-cultivated overnight in Luria Broth were

spread in a straight line in the center of a 90 mm Petri dish and two agar plug of 5 mm diameter with growing mycelium of one of the fungal strains was placed to the opposite edges of the plates. The plates were sealed with parafilm, and incubated at 28°C until mycelia in the untreated controls reached the center of the plates. Inhibition (mm) of mycelial growth of the tested fungi was determined and compared with the mycelial growth of the untreated control plates.

The production of volatile metabolites with antifungal activity released by bacterial and yeast isolates was also investigated. Each bacterial or yeast strain (overnight cultures) was inoculated on a 55 mm LB or PDA plate, respectively, and incubated at 28℃ for 3 days. Then, the lids of these plates were replaced by the bottom of a PDA plate inoculated with a fresh fungal mycelial plug. Plates were sealed together with sticky tape to minimize gas exchange and further incubated for 5 days. Controls were prepared in a similar manner but the bottom plate contained no bacteria. In all these experiments, growth inhibition (mm) of the tested fungi was determined and compared with the fungal growth of the untreated control plates. At least three independent repetitions of each test have been conducted.

Antagonistic activity on wheat grain

Biocontrol activity of antagonistic bacterial/yeasts isolates was evaluated against mycotoxigenic fungi. Surface-sterilized wheat grains (10 g) were treated with 1 ml of antagonist (bacteria/yeast) suspension at concentration of 10^8 cells/ml (10^7 cells/g of wheat grains). Non-treated wheat grains served as controls. After 24 h, treated and non-treated wheat grains were inoculated with 1 ml of 10^5 conidia (10^4 conidia/g of wheat grains) of the tested fungi. After 7 days of incubation at 28°C, inhibitory activity of bacterial/yeast isolates was evaluated as follows: grains (5 g) were suspended in 50 ml phosphate-buffered saline with Triton (PBS-Triton) and incubated in a rotary shaker (160 rpm) at 28°C for 30 min. Then, 100 µL of serial 1/10 dilutions were plated on PDA plates supplemented with chloramphenicol (20 mg/ml), followed by incubation at 28°C up to for 4 days for the assessment of fungal populations. Fungal populations were expressed as colony-forming units (CFUs) per gram dry weights of wheat grains.

Bacterial growth on a semi-commercial scale

The selected bacterial strains were grown overnight in LB agar plates at 37°C. A single colony of each strain was taken with an inoculating loop and used to inoculate a primary subculture of 10 ml LB medium in a 30-ml Erlenmeyer flasks, which were grown overnight in shake cultures at 37°C. The experimental flasks, each consisting of 1000 ml

LB in a 5-liter Erlenmeyer flask, were inoculated with 1ml of this 18-h-old culture. Growth was carried out on an orbital shaker, overnight at 37°C.

Biocontrol activity of selected bacterial strains against mycotoxigenic fungi on wheat grains

Surface disinfected (as described earlier) seeds of wheat (500 g) were inoculated in 4-liter glass beaker with 50 ml of cell suspensions (overnight cultures; OD = 1.00) of each bacterial isolate that were selected according to their inhibitory effect on the fungal growth. Grains received sterile saline instead of bacterial inoculum were used as negative controls. 24 h after bacterial inoculation, 50 ml of 10^5 conidia/ml (10^4 conidia/g of wheat grains) of *A. flavus* and/or *F. proliferatum* was inoculated on wheat grains. Untreated wheat grains were also inoculated with fungal conidia as positive controls. Beakers were incubated at 28℃ for 7 days and disease development was assessed visually. For each bacterial treatment of seeds inoculated with each fungal isolate, at least three independent experiments have been conducted. The antifungal activity of bacterial strains on inoculated wheat grains was evaluated using the plate counting method and expressed as fungal colony forming units (CFUs)/g dry weight of wheat grains. Briefly, grain samples (10 g) were suspended in 50 ml phosphate-buffered saline containing 0.05% Triton X-100 (Sigma) and incubated with shaking (160 rpm) at 28°C for 30 min, then 100 µl of serial dilutions of the suspension were plated on PDA plates supplemented with chloramphenicol, followed by incubation at 28°C for 4 d, for counting of fungal CFUs.

Effect of *Bacillus amyloliquefaciens* on *A. flavus* spore germination. The spores of *A. flavus* were harvested from a PDA culture in sterile water, filtered to remove hyphae and adjusted by counting in a hemocytometer to 2×10^5 spores/mL in PDB medium. *B. amyloliquefaciens* was selected according to its inhibitory effect on the mycelial growth of *A. flavus*. Two to three bacterial colonies grown on LB agar medium were inoculated in PD broth and incubated overnight at 28℃ under 180 rpm shaking. The overnight bacterial liquid culture was diluted to OD_{595} = 1 in PDB. Nine µL of bacterial culture were mixed with 3 µL of *A. flavus* spores at a final concentration of 5×10^4 spores/mL on a glass slide. Controls contained 9 µl of PDB medium instead of bacterial culture. The glass slides were placed in a humid box and incubated for 24h. The germination of spores was observed using Olympus BH2 microscope with bright-field settings. At least 30 spores of *A. flavus* were analyzed. The analysis criterion was the percentage of germinated spores.

Aflatoxin degradation assays. Commercial standards of aflatoxin B1 (AFB1) and deoxynivalenol (DON) were purchased from Fermentek (Jerusalem, Israel). AFB1 and DON were dissolved at a concentration of 1mg/ml (each) in methanol and stored at −20℃. Since reduction of mycotoxin accumulation in *Rhodotorula glutinis*-treated stored pome fruits has been reported previously (Castoria et al., 2005), the yeast *R. glutinis* strain Ty1, which was isolated from stored wheat grains, was selected for the mycotoxin degradation assay. The analysis of AFB1 and DON degradation in PD broth (PDB) medium was performed as follows. *R. glutinis* sp. TY1 was grown overnight in 50 ml of PDB medium in shake cultures at 28°C. The culture was centrifuged for 5 min at 8,000 × *g*, the cells were resuspended in PDB, and their concentration was adjusted to 1 × 10^8 CFU/ml. Twenty milliliters of this suspension was incubated on an orbital shaker for 3 days at 28°C in 100-ml flasks in the presence of 250 ng/ml of AFB1 and/or 4000 ng/ml of DON. Controls were uninoculated PDB medium with above mentioned mycotoxin concentrations, and PDB without mycotoxins inoculated with *R. glutinis* TY1 at the same cell concentration as reported above. Growth was monitored on a daily basis by reading the OD at 595 nm using a NanoDrop One^c spectrophotometer. At the same time points, the time course of AFB1 and/or DON degradation was monitored by high-performance liquid chromatography (HPLC).

AFB1 degradation by *R. glutinis* TY1 isolate was also examined in stored wheat grains. Briefly, surface disinfected (as described earlier) seeds of wheat (10 g) were amended with AFB1 (final concentration 250 ng/g) and inoculated with TY1 strain (final concentration 1 × 10^8 cells/g) on Petri dishes. Controls consisted of uninoculated wheat grains with and without AFB1 and TY1-inoculated grains without AFB1. The plates were prepared in triplicate and kept at 28°C up to 3 days. Then, the grains samples were frozen in liquid nitrogen, freeze dried and milled into a fine powder with a grain grinder. AFB1 was extracted after 24, 48, and 72 h and samples were analyzed quantitatively by HPLC. Experiments were performed three times.

HPLC analysis. For AFB1 and DON extraction from PDB broth, the samples were centrifuged at 8,000 × *g* for 5 min for pelleting the cells, and 2 ml of the supernatant was mixed with 2 ml of chloroform and vortexed for 15 min. After discarding the upper phase, the chloroform phase was dried at 50°C under a stream of gaseous nitrogen. For AFB1 analysis from wheat grains, 2.5 g of the ground seeds were mixed with 10 ml of 84:16 (v/v) acetonitrile:water and placed in an orbital shaker at 200 rpm for 1 h at room temperature. After centrifugation at 8,500 × *g* for 15 min, 2 ml of the supernatant were

passed through the Supel^{TM} TOX AflaZea column (SUPELCO, Bellefonte, PA, USA); then, the collected sample was evaporated under a stream of gaseous nitrogen at 50°C. The dried samples were reconstituted in 900 µl of 10% acetonitrile and 100 µl of trifluoroacetic acid solution (70% water, 20% trifluoroacetic acid and 10% acetic acid) for 15 min at 50°C. After incubation, the samples were filtered using 0.22 µm PTFE membrane filter, and quantitatively analyzed by injection of 30 µl into reverse phase HPLC/UHPLC system (Waters ACQUITY ^{Arc}, USA) with a gradient elution of 70% water, 15% acetonitrile and 15% methanol at 1 ml/min through a Kinetex 3.5 µm XB-C_{18} (150 x 4.1 mm) with (Phenomenex, USA). The column temperature was maintained at 35°C. Three non-contaminated PDB broth and/or wheat grain samples were spiked with AFB1 standard solution at three concentration levels for construction of calibration curves, which were used for mycotoxin quantification. AFB1 peak was detected with a fluorescence detector (excitation at 365nm and emission at 455 nm) and quantified by comparing with calibration curves of the standard mycotoxin.

Results

Epiphytic and endophytic wheat grain microbiome composition

Alpha diversity comparisons based on Shannon index, indicated that diversity of both bacterial and fungal epiphytes varied significantly ($P < 0.003$) compared to endiphytic communities (Fig. 1).

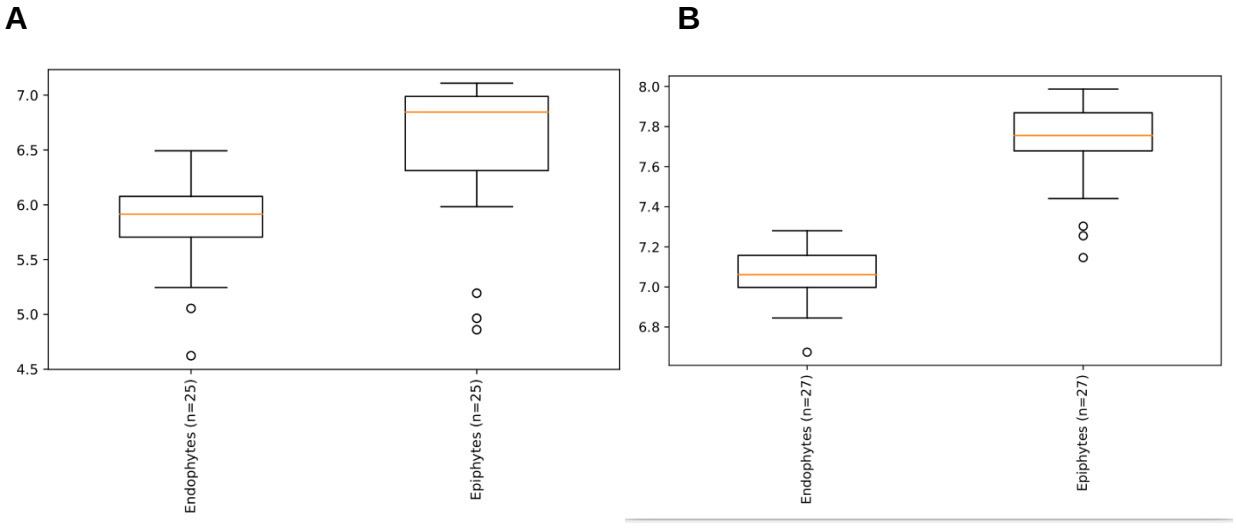


Fig. 1. Alpha diversity analysis (based on Shannon diversity index) of the bacterial (A) and fungal (B) epiphytic and endophytic communities in stored wheat grain samples.

Based on the Bray Curtis dissimilarity metric, both epiphytic and endophytic populations, varied significantly ($P < 0.01$) in their bacterial and fungal community composition and structure. The differences between epiphytic and endophytic communities were also

evident in the Principal Coordinate Analysis (PCoA) of the bacterial (Fig. 2A) and fungal (Fig. 2B) populations.

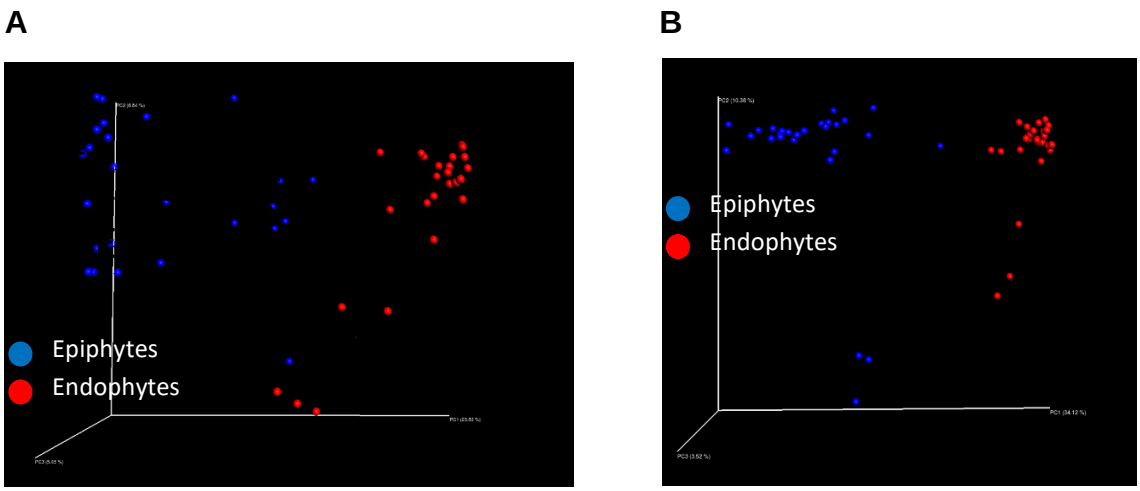


Fig. 2. Principle coordinate analysis (PCoA) based on beta diversity Bray Curtis dissimilarity metrics, showing the distance in the bacterial (A) and fungal (B) communities between epiphytes and endophytes

The relative abundance of bacterial and fungal genera detected in wheat grain samples among epiphytes and endophytes is shown in Fig. 3. Epiphytic bacterial communities of stored grains mainly consisted of genera of *Pantoea* (20.3%), followed by *Sphingomonas* (10.2%), *Massilia* (8.5%), *Pseudomonas* (8.1%), *Microbacteraceae* (4.3%), and *Curtobacterium* (4.3%) (Fig. 3A). *Pantoea, Bacillus, Pseudomonas, Ohtaekwangia,* unidentified SG8-4, *Enterobacter* and *Actinobacteria* were the most abundant bacterial genera found among endophytes. The relative abundance of several other bacterial taxa, varied significantly between epiphytes and endophytes (Fig. 3A). The most abundant epiphytic fungal genera consisted of *Alternaria* (50.3%), *Cladosporium* (10.5%), *Sporobolomyces* (9.5%), *Filobasidium* (8.8%) and *Stemphylium* (3.5%); endophytic fungal communities mainly consisted of *Alternaria* and *Stemphylium*, which accounted around 90% of the total endophytic fungal community (Fig. 3B).

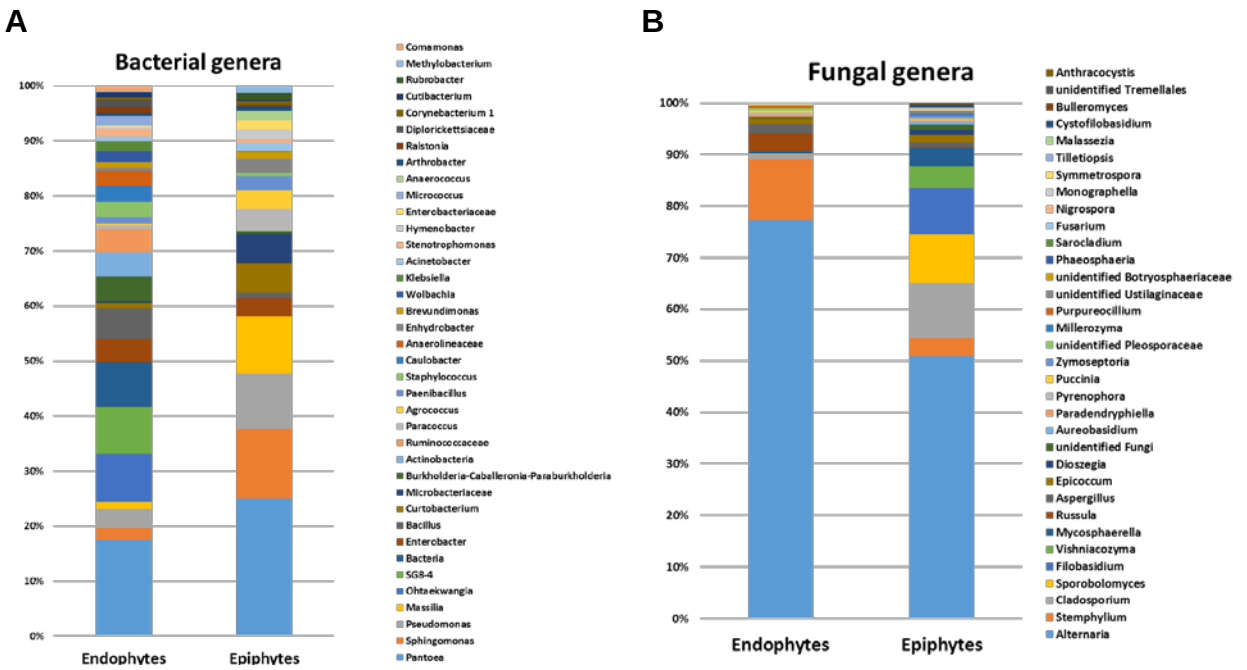


Fig. 3. Relative abundance of bacterial (A) and fungal (B) genera among endophytes and epiphytes detected in stored wheat grain samples using high-throughput sequencing technology (total of 21 samples collected during the experiment).

Isolation and identification of bacterial and fungal isolates from epiphytic and endophytic microflora of wheat grains

A total of 165 bacterial isolates were cultured from seeds; among them, 118 bacterial isolates were isolated from epiphytic microflora of the grains and 47 isolates cultured from endophytic microbiome (Fig. 4A). *Bacillus* spp. were predominant among cultured bacterial epiphytic community (53.4%) followed by *Pseudomonas* spp. (10%) and *Pantoea* spp. (6.8%). Similar trend has been observed among endophytic bacterial population, where *Bacillus* spp. were most common (46.8%), followed by *Pseudomonas* spp. (12.7%), *Pantoea* spp. (6.4%) and *Paenibacillus* spp. (6.4%) (Fig. 4A). A relatively diverse fungal community was identified from stored wheat grains (a total of 141 isolates) (Fig. 4B). Hundred and four (104) epiphytic isolates cultured from wheat grains and identified as *Alternaria* spp. (27%), *Fusarium* spp. (5.7%), *Aspergillus* spp. (5%), and *Penicillium* spp. (2.5%). *Cryptococcus*, *Filobasidium* and *Rhodotorula* species predominated among the isolated yeasts epiphytic cultures (27%) based on morphological features and sequencing analysis. The 37 endophytic fungal isolates cultured from wheat grains and were identified as *Alternaria* spp. (37.8%), *Fusarium* spp. (13.5), spp. and *Aspergillus* spp. (8%). *Filobasidium, Cryptococcus* and *Rhodotorula* spp. were still the most abundant (35%) yeast taxa among endophytes (Fig. 4B).

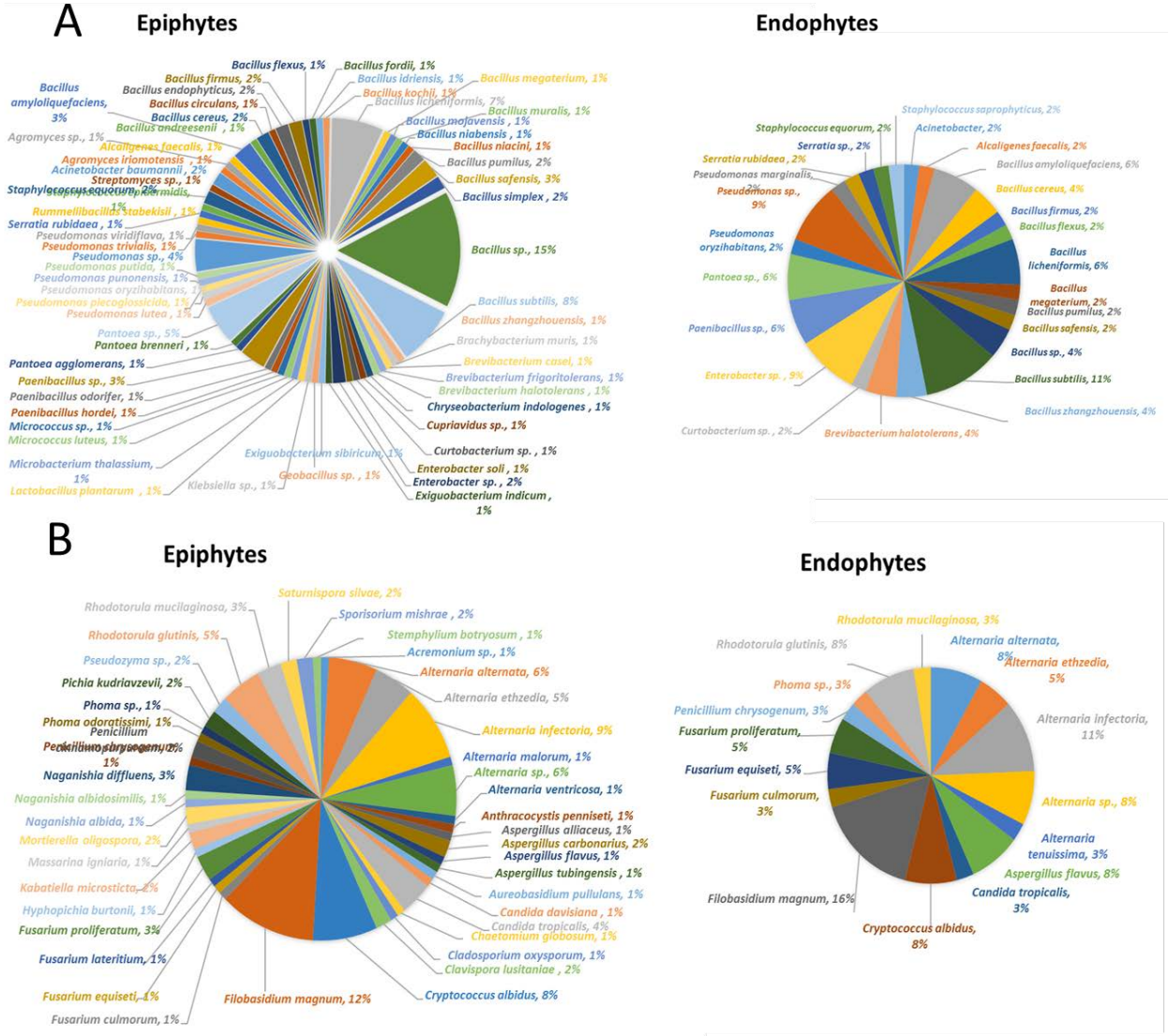


Fig. 4. Relative abundance of bacterial (A), fungal and yeast (B) genera isolated from epiphytic and endophytic microflora of wheat grains. The isolates were identified by sequencing the 16SrRNA gene in bacteria, ITS and D1/D2 regions in molds and yeasts, respectively. The sequences were determined via BLAST matches to the NCBI database

Analysis of mycotoxigenic fungi in stored grains by real-time PCR

Real-time PCR method was utilized to quantify fungal DNA in stored grain samples. The broadspectrum primers and specific fluorescent probes were used to amplify the targeted genomic DNA and to detect and quantify the major mycotoxigenic fungal genera, *Aspergillus*, *Fusarium*, and *Alternaria*. The wheat grain samples collected throughout the entire study did not exhibit any evidence of spoilage. Nevertheless, the qPCR analysis indicated the presence of all three pathogenic genera in majority of the samples, which corroborates the results obtained in the culture assay of wheat grains. Nine to eleven out of 21 wheat grain samples were found to be positive for all three genera by qPCR, with a relatively higher *Alternaria* DNA content (8-30 ng/mg seeds) relative to the other tested mycotoxigenic species. The obtained Ct (cycle threshold) values for *Aspergillus* and

Fusarium DNA samples ranged between 27.62 and 32.78, and indicated the presence of relatively low concentrations of the fungal DNA in the samples (2-7 ng/mg seeds). This suggests that qPCR is more sensitive in detecting mycotoxigenic fungi compared to culture-based approaches.

Antifungal activity of selected antagonistic bacterial/yeast isolates

All bacterial and yeast isolates cultured from epiphytic and endophytic microflora of wheat grains were tested for the potential antagonistic activity against mycotoxigenic fungi. Only four bacterial and four yeast isolates exhibited antifungal properties against mycotoxigenic fungi, such as *A. flavus, A. infectoria* and *F. proliferatum* (Table 1).

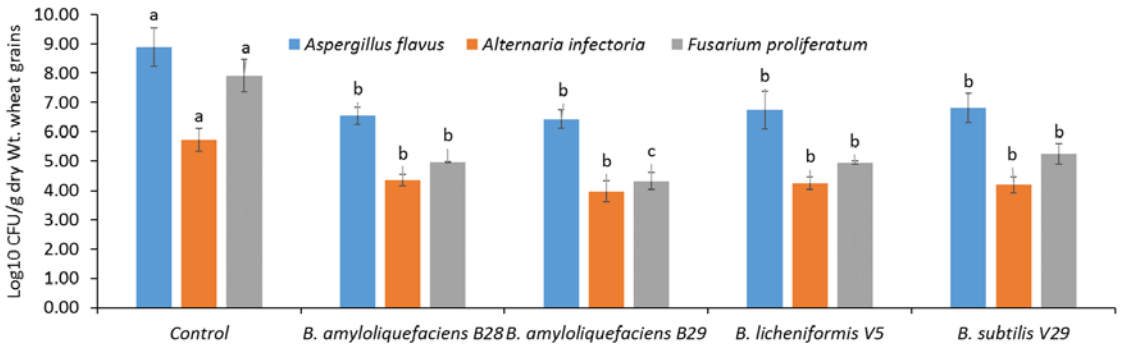
Table 1. Bacterial and yeast isolates with antifungal activity

Yeasts (identified based on D1/D2 region sequencing)	**Source**
Naganishia albidosimilis D1	Epiphyte
Naganishia albida D34	Epiphyte
Cryptococcus albidus D37	Epiphyte
Rhodotorula glutinis Ty1	Epiphyte

Bacteria (identified based on *rpoB* gene sequencing)	**Source**
Bacillus amyloliquefaciens B28	Endophyte
Bacillus amyloliquefaciens B29	Endophyte
Bacillus licheniformis V5	Epiphyte
Bacillus subtilis V29	Epiphyte

In vivo experiments in wheat grains under laboratory conditions showed that all tested isolates were more effective against *A. infectoria* and *F. proliferatum* than against *A. flavus* (Fig. 5).

A



B

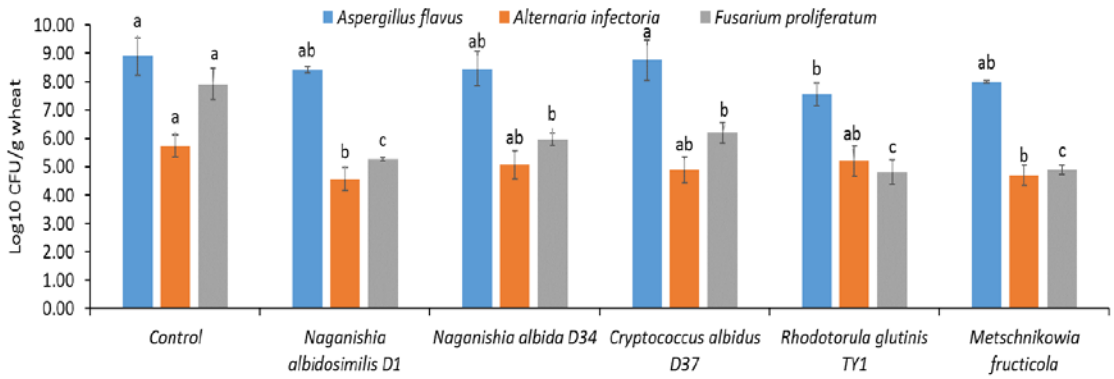


Fig. 5. *In vivo* inhibitory effect of selected bacterial (A), and yeast (B) strains, isolated from epiphytic and endophytic microflora of stored wheat grains, against mycotoxigenic fungi. Error bars represent the standard error of the mean (SEM) across three independent replicates. One-way ANOVA differences were considered significant when $p < 0.05$. Different letters above the error bars indicate statistically significant differences as determined based on Duncan's multiple range test.

Among the tested isolates, endophytic bacterial strain *Bacillus amyloliquefaciens* (B29) found to be the most effective against all representative mycotoxigenic fungi. This isolate significantly inhibited fungal growth in stored wheat grains compared to untreated controls (Fig. 5A).

Large-scale trials for testing of biocontrol activity of selected bacterial strains against mycotoxigenic fungi on wheat grains

In the next step, four selected bacterial strains, which used in previous experiments, were grown at semi-commercial scale (see "Materials and methods"). Subsequently, the bacteria were applied to large-scale samples (500 g) of wheat grains.

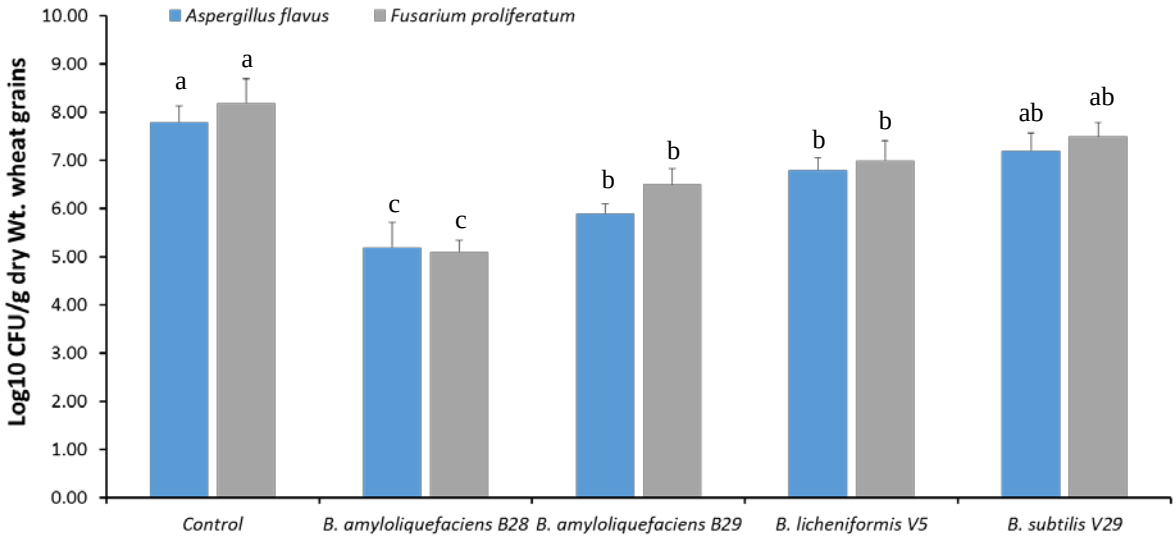


Fig. 6. *In vivo* inhibitory effect of selected bacterial strains, which grew on a semi-commercial scale, against mycotoxigenic fungi in stored wheat grains. Error bars represent the standard error of the mean (SEM) across three independent replicates. One-way ANOVA differences were considered significant when $p < 0.05$. Different letters above the error bars indicate statistically significant differences as determined based on Duncan's multiple range test.

As shown in Fig. 6, bacterial strain *Bacillus amyloliquefaciens* (B28) found again to be the most effective antagonist against the major mycotoxigenic fungi, *A. flavus* and *F. proliferatum*. Relative to other tested bacterial strains, this isolate significantly inhibited fungal growth in stored wheat grains compared to untreated controls (Fig. 6).

B. amyloliquefaciens strongly affected spore germination of *A. flavus*

To characterize the antifungal potential of this bacterial strain beyond mycelial growth inhibition, its ability to interfere with spore germination was assessed by co-inoculating

A. flavus spores with the bacterial cells. Spore germination was observed in untreated controls after 24h; but the pathogen's germination rate was severely reduced (to 20%) in samples exposed to *B. amyloliquefaciens* bacterial cells.

Mycotoxin-degrading activity of the yeast *Rhodotorula glutinis* strain TY1 isolated from stored grains

Aflatoxin B1 (AFB1) and deoxynivalenol (DON) degradation by *R. glutinis* TY1 strain was examined by inoculating the yeast in PDB medium with the presence of AFB1 and/or DON (Fig. 7).

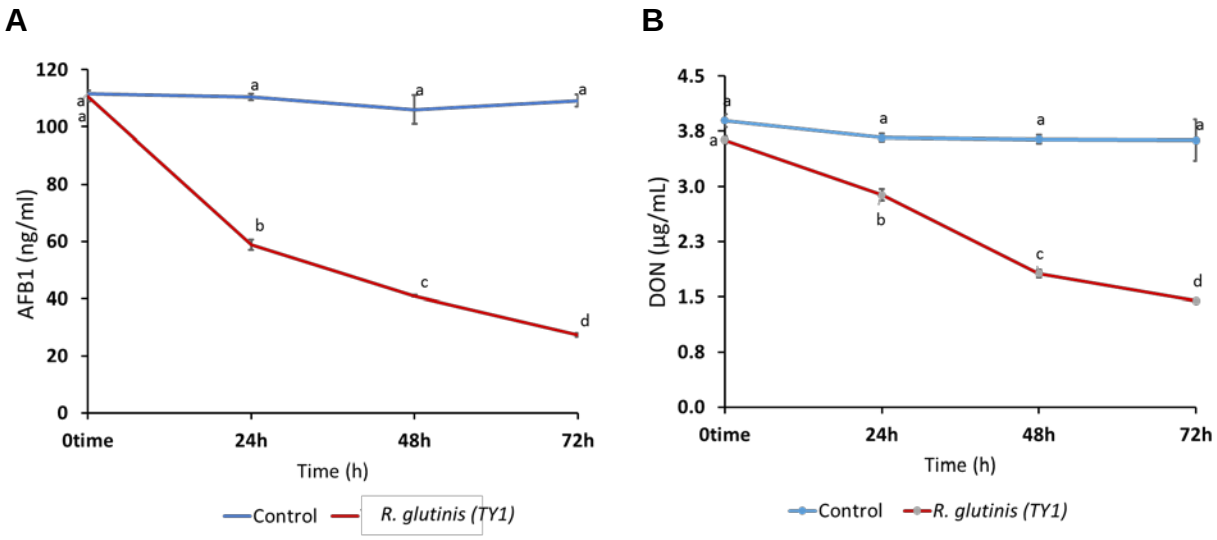


Fig. 7. Effect of *R. glutinis* TY1 strain on AFB1 (A) and DON (B) degradation in PDB media. The time course of AFB1 and/or DON degradation was monitored by HPLC. Error bars represent the standard error of the mean (SEM) across three independent replicates. One-way ANCOVA was conducted to determine a statistically significant difference between treatment and control on mycotoxin concentration controlling for time (covariance). Time significantly differed in its effect on treatment and control ($P<0.0001$). Testing for each time point using ANOVA is an evident that only time zero does not differ between control and treatment. Different letters above the error bars indicate statistically significant differences at $p < 0.05$, as determined using the Duncan's multiple range test.

Fig. 7 reports the quantitative results of AFB1 (Fig. 7A) and DON (Fig. 7B) in time-course HPLC analyses. In the presence of viable yeast cells, 3.6-fold and 2.6-fold reduction of AFB1 and DON levels, respectively, was observed by the end of the experiment (72h). These results demonstrate that strain TY1 can degrade both AFB1 and DON *in vitro*. Yeast cells applied in wheat grains are able to degrade AFB1 (Fig. 8); almost 3-fold reduction in AFB1 concentration was observed in *R. glutinis* treated seeds compared to untreated control. No degradation activity of *R. glutinis* on DON was observed in inoculated seeds (data not shown).

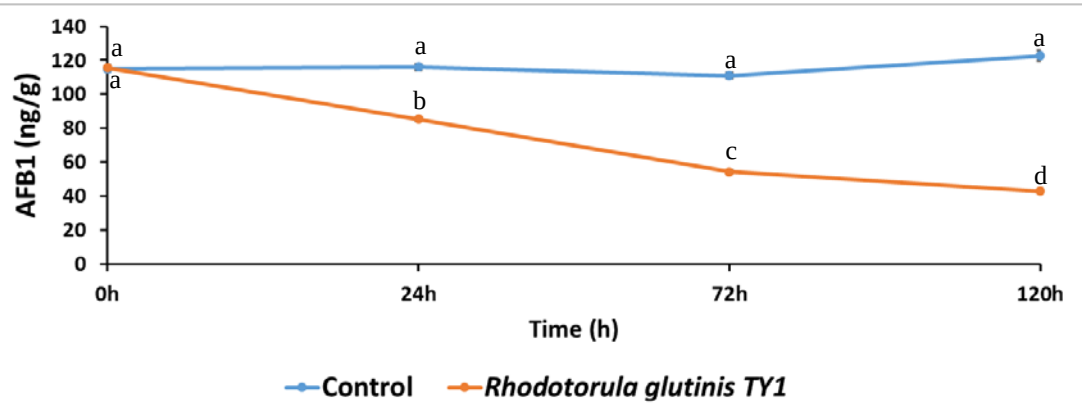


Fig. 8. Effect of *R. glutinis* TY1 strain on AFB1 degradation in wheat grains. The time course of AFB1 degradation was monitored by HPLC. Error bars represent the standard error of the mean (SEM) across three independent replicates. Different letters above the error bars indicate statistically significant differences at $p < 0.05$, as determined using the Duncan's multiple range test. One-way ANCOVA was conducted to determine a statistically significant difference between treatment and control on AFB1 concentration controlling for time (covariance). Time significantly differed in its effect on treatment and control ($P<0.0001$).

These data suggest that yeast cells with potential biocontrol activity could degrade mycotoxin and/or negatively affect its accumulation or synthesis in wheat grains.

Discussion

Characterizing the microbiota of wheat grains after harvest is an essential step to understanding the interactions that potentially occur between different members of the community in relation to the colonization of the grains with common mycotoxigenic fungi (*Aspergillus*, *Fusarium* and *Alternaria* species). It is known that many factors could affect the stored seed microbial community such as temperature, humidity, water activity, grain moisture (Schmidt et al., 2018). Nevertheless, in the current study, the impact of such environmental factors was minor due to the optimal and stable weather conditions during storage period. The results of the meganomic analysis, obtained during the first year of the project, indicated that fumigation of the grains with phosphine had a significant effect on the diversity and abundance of various components of the wheat-grain microbiome. In general, the composition of the bacterial and fungal microbiota of wheat grains found in the current study is in agreement with previous studies (Gdanetz and Trail, 2017; Comby et al., 2016). Genera, including *Bacillus, Erwinia, Pseudomonas,* and *Paenibacillus* were dominant bacterial taxa identified, while *Alternaria*, *Stemphylium*, *Cladosporium*,*Sporobolomyces*, *Mycosphaerella*, and *Filobasidium* were the most prevalent fungi and represent the most common members of the wheat grain mycobiome.

Since phosphine is toxic to aerobically respiring organisms, it also has effects on the survival and growth of some aerobic bacteria and fungi (De Castro et al., 2001; Hocking

and Banks, 1991). In the present study, bacterial population exhibited a drastic reduction in their level of diversity and the number of observed species after fumigation. This change corresponded with a significant increase in *Bacillus* from an average of 2.2% before fumigation to more than 50% after fumigation. Results indicated that neither the diversity nor abundance of fungi were affected by phosphine fumigation (Solanki et al., 2019). Only a few studies have described the influence of phosphine on the survival and growth of molds in stored grains. A reduction of *A. flavus* growth and aflatoxin production was also observed in response to phosphine treatment of maize kernels stored at different moisture levels (De Castro et al., 2001). In the same study, however, the authors stated that *Penicillium* species and *F. verticillioides*, which are commonly found in freshly harvested grains, were tolerant to phosphine (De Castro et al., 2001). Those findings are consistent with the results obtained in the current study of wheat-grain-associated microflora, in which a greater number of *Fusarium* species, such as *F. culmorum* and *F. proliferatum*, were detected at the last sampling time point, probably due to the changes that occurred in microbial community composition following phosphine fumigation. Notably, the presence of mycotoxigenic *Fusarium* isolates in the stored wheat grains after fumigation was strongly associated with the occurrence of the mycotoxin DON (deoxynivalenol) in samples collected at the last time point, suggesting that phosphine induced a shift in the microbial composition towards more toxigenic strains.

During the second year the research focused on identification of epiphytic and endophytic microbial communities of stored wheat grains using high-throughput sequensing technology and traditional culturing methods of isolation. The results obtained with the culturing assay of stored wheat grains microbes were in agreement with the results obtained by high-throughput sequencing data. *Bacillus, Pseudomonas*, and *Pantoea* were found to be most abundant genera obtained in the cultured bacterial taxa, while *Alternaria*, *Fusarium, Cryptococcus* and *Filobasidium* were the most prevalent fungi isolated from stored wheat grains throughout the study, and the same was true in the high-throughput sequencing analysis. These microorganisms represent the most common members of the wheat grain epiphytic and endophytic microbiome. Selected species isolated from stored wheat grains, such as *Bacillus* spp., and yeast isolates such as *Naganishia* spp., *C. albidus* and *R. glutinis* were found to be effective *in vitro* and *in vivo* against mycotoxigenic fungi. *Bacillus* spp. showed significant antagonistic activity in grains against *A. flavus*. *F. proliferatum* and *A. infectoria*, while selected yeast isolates, such as *R. glutinis*, showed their ability to degrade mycotoxins, suggesting that

mechanism of bacterial antagonistic activity is different from that of yeasts. We considered of interest to validate our results regarding biocontrol potential of *Bacillus* isolates in a setting in which the large-scale experiments could be performed. As depicted in Fig. 6, the growth of *A. flavus* and *F. proliferatum* on the seeds was markedly restrained when wheat seeds were pretreated with cell suspension of endophytic isolate of *B. amyloliquefaciens* strain B28. Our results indicate that *B. amyloliquefaciens* isolated as endophyte from inner tissues of wheat grains, and which is already known for its antagonistic properties toward fungal pathogens (Baffoni et al., 2015; Crane and Bergstrom, 2014; Gong et al., 2015), might be promising as biocontrol agent against *Aspergillus* and *Fusarium* infections in stored wheat grains. An efficient biocontrol agent should not only inhibit mycelial growth but also prevent spore formation or germination. We thus tested whether the potential biocontrol isoalte showing high inhibition of *A. flavus* mycelial growth would also inhibit spore germination. This was indeed the case for *B. amyloliquefaciens* strain B28, which had a strong inhibitory effect on *A. flavus* spore germination. As mentioned in "Materials and Methods", the production of volatile metabolites with activity against fungal growth was also investigated. However, no effect attributable to volatile substances could be proved (data not shown). Furthermore, this study demonstrates that *R. glutinis* B28 yeast isolate appears to degrade mycotoxins *in vitro* and in a stored wheat-grain model system. This yeast isolate is able to decrease AFB1 toxin concentration in inoculated grains. Such decrease is possibly due to metabolization of the mycotoxin by *R. glutinis* yeast cells, but the mechanism is to be confirmed.

In summary, we have identified the composition of the stored wheat-grain-associated epiphytic and endophytic microbiota, using high throughput sequencing technology. The development of next generation sequencing technologies have provided researchers a deeper understanding of the microbiome on plant material, and gave an opportunity to reveal large numbers of microbes generating communities that can affect disease outcomes. Large numbers of microorganisms forming epiphytic and endophytic communities often possess attractive characteristics, including antagonistic activity against fungal pathogens. Indeed, this work has shown that stored wheat seeds harbor bacterial and yeast antagonists with strong inhibition potential and biodegradation ability against mycotoxigenic fungi and their respective toxins. Application of such microbes, which adapted to their host plant, might contribute to efficient fungal disease control strategies in stored grains and other agricultural commodities as well. A better

understanding of antagonistic mechanisms of these microorganisms may assist in the development of novel antifungal biocontrol approaches to replace the traditionally used synthetic fungicides.

Nevertheless, based on the findings of the present study, we continue to develop biocontrol agents as part of a new project funded by the Chief Scientist (since 2020, Tevat Noah). Microorganisms with effective antagonistic properties will be developed into formulations with the aim of using them on a commercial scale against mycotoxigenic fungi and mycotoxins in cereals.

Acknowledgments

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