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אסטרטגיות חדשות למניעת התפתחות פטריות מיקוטוקסיגניות ויצירת מיקוטוקסינים בגרעיני חיטה למאכל וגרעיני מספוא

New strategies to prevent the development of mycotoxigenic fungi and mycotoxins in wheat grain for food and feed

מוגש לקרן המדען הראשי במשרד החקלאות ע"י

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תקציר

מחזור שנתי של גרעיני חיטה למאכל וגרעינים וקמחים למספוא בישראל מסתכם בכ-4.5 מיליון טון כאשר ערכם כספי מגיע ל-1.5 מיליארד דולר. זיהום פטרייתי הוא אחד מהגורמים העיקריים לקלקול גרעיני חיטה למאכל ולמספוא. זיהומים אלה גורמים להפסדי תבואה ניכרים וכתוצאה מכך לנזקים כלכליים כבדים. פטריות עובש מיקוטוקסיגניות מהוות סיכון גבוה לבריאות האדם ובעלי החיים עקב יכולתן לייצר מיקוטוקסינים אשר הוכחו כמסרטנים וגנוטוקסיים.

מעקב וניטור מהיר של פטריות מיקוטוקסיגניות ומיקוטוקסינים במחסני חירום של גרעיני חיטה למאכל ולמספוא מבוצע תוך פיתוח ויישום של שיטות ביולוגיה מולקולרית וכימיה אנליטית. ניתוח מיקרופלורה אפיפיטית של גרעיני חיטה בוצע באמצעות אנליזת DNA שהופק מתערובות של גרעיני חיטה וריצוף של 16S, ITS ו-18S RNA גנים ריבוסומליים הנחוצים לתאים פרוקאריוטיים ואאוקאריוטיים, בהתאמה; ניתוח התוצאות בוצע בשיטת ריצוף עמוק (deep sequencing) בכדי לזהות תבדידים אנטגוניסטיים לפטריות עובש. כמו-כן, בידוד וזיהוי של מיקרופלורה (חיידקים ושמרים) של גרעיני חיטה נעשה בהצלחה באמצעות שיטות מורפולוגיות ומולקולריות.

הסקר יסייע לאיסוף מידע על מימדי הבעיה במחסני חירום וגם ללימוד המרכיבים השונים של אוכלוסיית פטריות העובש. התוצאות שהתקבלו עד כה מצביעות על נוכחות של פטריות מיקוטוקסיגניות, בעיקר מסוגים של *Aspergillus*, *Alternaria* ו-*Fusarium*, במספר דגימות של גרעיני חיטה מאוסמים; בכ-20% מהדגימות נמצאו מיקוטוקסינים מעבר לרמות מקסימליות המותרות עפ"י הרגולציה של האיחוד האירופי. מספר מיקרואורגניזמים שבודדו מפלורה אפיפיטית של גרעיני חיטה (בעיקר חיידקי *Bacillus*) הראו פעילות אנטיפטרייתית in vitro. תוצאות החקר וניתוח של מיקרופלורה של גרעיני חיטה

מאוחסנים עלולות להוביל לפריצת דרך במציאת תבדידים אנטגוניסטים ידידותיים לסביבה כאלטרנטיבה לתרכובות כימיות כנגד מגוון רחב של פטריות מיקוטוקסיגניות.

רשימת פרסומים והרצאות שנבעו מהמחקר:

1. Sadhasivam, S., Britzi, M., Zakin, V., Kostyukovsky, M., Trostanetsky, A., Quinn, E., Sionov, E. (2017). Rapid detection and identification of mycotoxigenic fungi and mycotoxins in stored wheat grain. *Toxins*. 9: 302; doi: 10.3390/toxins9100302.
2. Solanki M.K. (2017). Analysis of wheat associated microbiota and its role in biocontrol of mycotoxigenic fungi. The Annual Meeting of the Israel Society for Microbiology. The Volcani Center, ARO.
3. Sionov E. (2017). Investigating the stored wheat grain microbiome for biological control of mycotoxigenic fungi. The Annual Meeting of the Israeli Society for Molecular Mycology (MMM). The Volcani Center, ARO.
4. Sionov E. (2018). Molds and mycotoxins in agricultural products and food – strategies to overcome it. The Annual Meeting of the Israeli Society for Medical Mycology. Tel-Aviv University.

Contents

Introduction

Wheat grain and associated by-products constitute important sources of energy and protein for human beings and all classes of farm animals. When grains are colonised by molds there is a significant risk of contamination with mycotoxins, which are low-molecular weight natural products produced as secondary metabolites by these fungi. Many species of *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* are not only recognised plant pathogens but are also sources of the important mycotoxins of concern in animal and human health (1). Poor postharvest management can lead to rapid deterioration in grain quality, severely decreasing germinability and nutritional value of stored grain, with possible undesirable fungal contamination and consequently toxin production (2). The most frequently detected mycotoxins in wheat grain are deoxynivalenol, fumonisins, and zearalenone, produced by *Fusarium* species, and aflatoxins and ochratoxins produced by *Aspergillus* and *Penicillium* species, respectively (3). Timely assessment of these contaminants as well as the identification of main toxicogenic fungal species is important not only for assessing food quality but also to develop control strategies for obtaining safe food (4). Surveillance and monitoring for mycotoxigenic fungi and mycotoxins becomes critical for maintaining high quality grains and grain products in indoor storage facilities. Molecular approaches such as PCR can serve as good alternatives to conventional methods for the detection of mycotoxigenic fungi. In addition, multi-mycotoxin determination methods are required due to their co-occurrence in different commodities (5). The complexity of wheat grain matrix, as well as the wide range of physical and chemical properties of mycotoxins, requires selective and sensitive detection techniques for multi-toxins. Since the occurrence of mycotoxigenic fungi and mycotoxins in Israeli stored wheat grain for food and feed has not yet been investigated, the aim of the project during the first year was to optimize and evaluate a reliable multiplex PCR assay and LC-MS/MS multi-method for simultaneous detection and accurate determination of the critically important mycotoxigenic fungi and mycotoxins, respectively, in these commodities.
Furthermore, protecting stored wheat grain from fungal spoilage is an essential part of their production. Wheat associated microorganisms can have beneficial effects on the stored grain's health. Understanding the composition and role of stored wheat grain microbiota is crucial toward agricultural practices that are less dependent on chemical fungicides, which has known negative effects on the environment and human health. Therefore, during the second year of the present study, we used 16S and 18S rRNA

high throughput sequencing to explore stored crop seeds microbial communities and also obtained a large number of bacterial and yeasts isolates from epiphytic microflora of wheat grains, which have been assessed for their antifungal activity in vitro and in vivo. The results indicate that some of the screened isolates presented antagonistic properties toward mycotoxigenic pathogens such as *Aspergillus*, *Fusarium* and *Alternaria* spp.

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

1. Surveillance and monitoring of mycotoxigenic fungi and mycotoxins in wheat grain storage warehouses
2. Analysis of epiphytic microflora of stored wheat grains in order to find genetically stable isolates and antagonists against a wide range of mycotoxigenic fungi
3. To test the efficacy of potential antagonistic isolates under various storage conditions

Materials and methods

Wheat grain samples. A total of eight wheat grain storage warehouses located all over Israel were sampled, 3-6 months after harvest. 4-5 samples aliquots of wheat grain for food (1 kg) were collected from the front face and in the middle of the warehouses, at 1 m horizontal depth, and in areas close to the walls. Grain temperature and moisture content were in the ranges of 27-33^{0}C and 10.5-12.9%, respectively. On the same day a 100 g aliquot from each thoroughly mixed sample was frozen in liquid nitrogen, lyophilized and milled into a fine powder using a grain grinder.

DNA Extraction. The DNA from wheat grain samples was extracted from milled grains; fungal genomic DNA was isolated from lyophilized mycelial mats grown overnight in YPD medium, as described previously (6).

Multiplex PCR. In order to optimize the multiplex PCR assay for direct detection of mycotoxigenic fungal species in naturally infected wheat grain samples, primers used previously for species-specific detection and for amplification of genes involved in mycotoxin biosynthesis (Tables 2, 3) were tested for referenced fungi and confirmed by performing monoplex PCRs. Standardizing the multiplex PCR was performed by empirically varying critical factors that affect multiplexing, such as primer concentrations, template amount and annealing temperature. Eight different sets of 4-6 pairs of primers were combined for multiplex PCR considering annealing temperature and amplicon size.

Mycotoxin extraction and analysis. Individual stock standard solutions of aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2), ochratoxin A (OTA), zearalenone (ZEA), deoxynivalenol (DON), fumonisins (FB_1, FB_2) and T-2 toxins (Fermentek, Israel) were made in methanol. From the individual stock standard solutions, a multi-toxin stock-standard mixture was prepared in order to determine calibration curves and limit of detection (LOD) in solvent and matrix. Standards and samples were analyzed by QTRAP 6500 LC-MS/MS system (Applied Biosystems, USA) equipped with electrospray ionization (ESI) source and high performance liquid chromatography (Nexera X2, Shimadzu, Japan). Three non-contaminated wheat grain samples were spiked using multi-mycotoxin standard solutions at three concentration levels. Extraction and analysis were performed as described above. The spiking experiments were performed in triplicate at three different time points. Validation parameters, such as repeatability, accuracy, LOD, limit of quantification (LOQ) and specificity, were determined.

Gene amplification and sequencing. 16S rDNA gene for bacteria and the 18S 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 (7) and utilized to evaluate the β-diversity and construct PCoA plots (8). 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 (9). 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 bacterial and fungal species from wheat grain. The wheat samples were analyzed for presence of bacterial and yeasts isolates, and potentially mycotoxigenic fungi. In order to isolate microbes from wheat grains, a 10 g sample of seeds was incubated in 90 ml of peptone water (0.1%). Seed samples were incubated with shaking (150 rpm) at 28°C for 1h, then 100 µl of serial dilutions of the broth were plated on LB agar plates for isolation of bacterial cultures, and on PDA media supplemented with chloramphenicol (10 µg/ml) for yeasts and fungal isolates. LB plates (for bacterial growth) were incubated at 37°C for 24h; PDA plates incubated at 28°C for 3 days. DNA was extracted from each fungal strain using a CTAB method (6), and from each bacterial strain using a Wizard genomic DNA extraction kit (Promega). Fungal isolates were identified by sequencing of the ITS rRNA gene region; bacterial isolates were determined by sequencing of amplicons using 16S rRNA universal primers. The sequence of nucleotide alignments obtained was referenced against the GeneBank database (http://www.ncbi.nlm.nih.gov/BLAST) using the nucleotide blast program.

Results

Specificity and sensitivity of multiplex PCR

In this study, a multiplex PCR assay was applied to allow simultaneous detection of a wide range of potential mycotoxin producing fungi in wheat grain storage warehouses in Israel. Toward this end, five sets of 4-5 species-specific primer pairs were composed for the molecular identification of the most frequent fungal species representing seven *Aspergillus* spp, nine *Fusarium* spp, and five *Penicillium* spp. that might be found in stored wheat grain (Table 1). The specificity of all the species-specific primers was assessed by performing multiplex PCR on the genomic DNA of standard fungal isolates, which resulted in complete amplification all target genes (Figure 1A). In addition, three sets comprised of 4-6 primers to amplify the genes, which are

associated with mycotoxin biosynthesis, were created for the detection of mycotoxigenic fungi in the present study (Table 2).

Table 1. Sets of species specific primers used in this study

Set no.	Species	DNA/Gene target	Amplicon size (bp)	Annealing temperature
I	*A. fumigatus*	*pep*	250	
	A. flavus	*pepO*	200	
	A. parasiticus	*caM*	430	60ºC
	A. tubingensis	*caM*	505	
	A. carbonarius	*caM*	371	
II	*F. graminearum*	RAPD[1] marker	400-500	
	F. culmorum	RAPD marker	570	
	F. poae	RAPD marker	220	55ºC
	F. sporotrichioides	*tri13*	332	
III	*F. verticillioides*	*caM*	578	
	F. avenaceum	RAPD marker	920	
	F. solani	*TEF-1α*	658	55ºC
	A. niger	*caM*	357	
IV	*F. proliferatum*	*caM*	585	
	F. oxysporum	*ITS*	340	
	P. expansum	*IDH*	480	55ºC
	P. digitatum	*Cyp*51	250	
V	*P. paneum*	*IDH*	482	
	P. verrucosum	*Otanps*	750	
	P. roqueforti	ITS1- 5.8S- ITS2	300	60ºC
	A. terreus	Topoisomerase II gene	386	

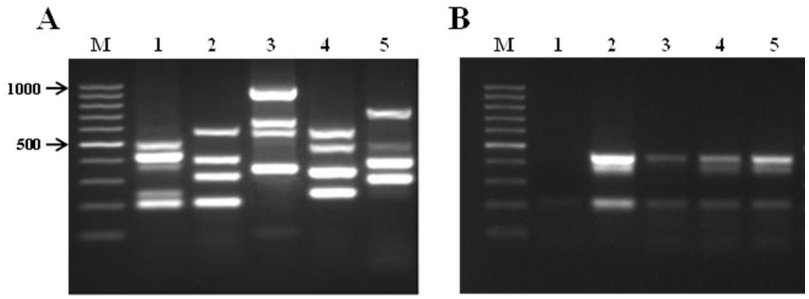
[1]RAPD – Random Amplified Polymorphic DNA

Table 2. Primer sets for genes involved in mycotoxin biosynthesis

Set no.	Mycotoxin	Gene target	Amplicon Size (bp)	Annealing temperature
VI	Aflatoxins	*aflR1*	798	
		nor1	397	
		avfA	950	58ºC
		ver1	452	
VII	Fumonisin/Trichothecene/ Zearalenone	*fum1*	798	
		fum13	988	
		tri5	450	55ºC
		tri6	546	
		pks13	351	
VIII	Ochratoxin A	*pks*	650	
		otanps	788	60ºC

The annealing temperature of each primer group for amplification of the target genes was set using gradient temperature conditions. To determine the minimum amount of fungal template necessary to obtain visible amplification products, the multiplex PCR assays were carried out using serial dilutions of fungal genomic DNA. The detection limit of purified genomic DNA was determined as 100 pg for all strains tested to produce a visible band on an ethidium bromide-stained agarose gel. When sensitivity of the multiplex PCR was evaluated by artificial inoculation of wheat grain samples with known amount of *Aspergillus* spp. conidia, the detection limit for each species was 1×10^4 spores per gram of sample, with no incubation, for DNA extracted directly from grains (Figure 1B).

Figure 1. Specificity and sensitivity of multiplex PCR assays using species specific primers

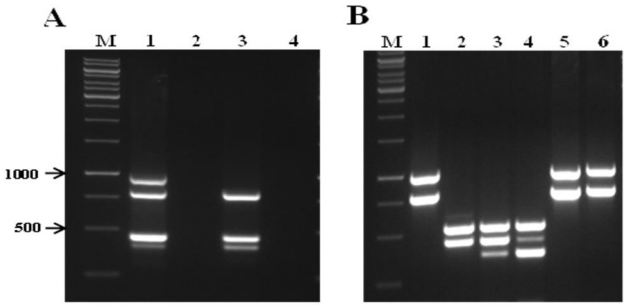


(A) Specificity test. **Lanes**: M - 100bp DNA ladder; 1 – primer set I; 2 – primer set II; 3 – primer set III; 4 – primer set IV; 5 – primer set V. **(B)** Sensitivity test. **Lanes**: 1 – negative control; 2 – positive control (wheat sample inoculated by *A. parasiticus* NRRL 6111, *A.fumigatus* NRRL 62427, *A. carbonarius* NRRL 368 and incubated for 48 hrs); 3-5 (represent samples inoculated by *A. parasiticus* NRRL 6111, *A.fumigatus* NRRL 62427, *A. carbonarius* NRRL 368 without incubation): 3 – 10^4 spores g^{-1}; 4 – 10^5 spores g^{-1}; 5 – 10^6 spores g^{-1}.

Application to stored wheat grain samples

Due to the high importance of wheat grain storage as part of the marketing, distribution and food security system, high quality and safety of this product need to be ensured. In order to characterize mycotoxin producing fungi by multiplex PCR, sets of the primers directed to the structural and regulatory genes involved in biosynthesis of aflatoxins (*aflR1*, *nor1*, *avfA*, *ver1*) and *Fusarium* toxins (*fum1*, *fum13*, *tri5*, *tri6*, *zea*) were tested using DNA extracted from different *Aspergillus* and *Fusarium* species, respectively (Figure 2A, B). The obtained results clearly show the presence of specific fragments amplified from DNA of isolates with potential mycotoxin-producing abilities. Some *Aspergillus* strains (*A. flavus* NRRL 3518, *A. flavus* SS2) did not show any amplification in all target genes (Figure 2A). The probability of a particular toxin being produced can be predicted by the presence or absence of an amplification product, but the effective biosynthesis of the toxin remains to be confirmed by analytical chemistry analysis (10,11).

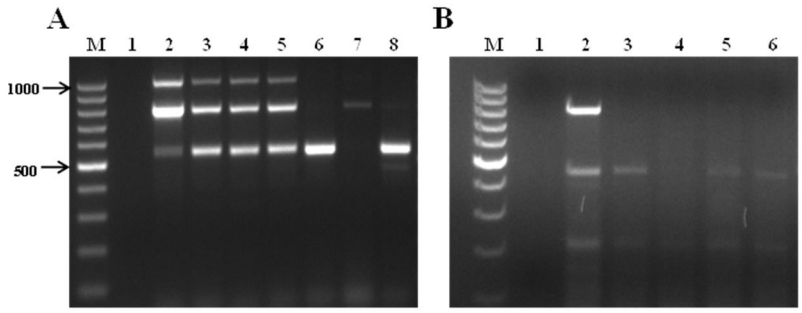
Figure 2. Specificity of multiplex PCR assays using sets of primers for mycotoxin biosynthetic genes



(A) Primer set VI. Lanes: M – 1kb DNA ladder; 1 – *A. parasiticus* NRRL 6111; 2 – *A. flavus* NRRL 3518; 3 – *A. flavus* SS1; 4 – *A. flavus* SS2. **(B)** Primer set VII. Lanes: 1 – *F. verticillioides* NRRL 25457; 2 – *F. sporotrichioides* NRRL 3299; 3 – *F. culmorum* NRRL 13320; 4 – *F. graminearum* NRRL 3376; 5 – *F. verticillioides* SS4; 6 – *F. verticillioides* SS5.

Primer sets for genes related to aflatoxin and *Fusarium* toxin biosynthesis were also tested on DNA samples obtained from wheat grain artificially infected by *Aspergillus* and *Fusarium* spp. These primers allowed us to identify potential aflatoxigenic, fumonisins and trichothecenes producing isolates in wheat grain. Since wheat grain could easily be contaminated before harvest and during post-harvest handling and storage by variety of mycobiota, multiplex PCR assays have been applied, and a total of 34 wheat grain samples collected from eight storage warehouses were tested for the presence of genes involved in biosynthesis of mycotoxins. The samples were examined directly using this method, without a fungus isolation and incubation step. Among the 34 grain samples twenty two showed positive signals using mycotoxin gene-based primer sets. Figure 3 shows some of the results obtained from a multiplex PCR using DNA extracted from wheat samples collected from two storage warehouses in northern Israel; in this case amplification products, have been obtained indicating the presence of potentially toxigenic *Fusarium* spp. (Figure 3A) and aflatoxigenic *Aspergillus* isolates (Figure 3B). There was no amplification in the clean non-contaminated sample that was used as a negative control. These results suggest that this method can be used as a diagnostic tool, which may allow the rapid and cost-effective detection of potential mycotoxigenic fungi in stored wheat grain.

Figure 3. Multiplex PCR using DNA isolated from stored wheat grain



(A) Primer set VII. Lanes: M – 100bp DNA ladder; 1 – Negative control; 2-8 – wheat grain naturally contaminated by toxicgenic *Fusarium* spp. **(B)** Primer set VI. Lanes: 1 – Negative control; 2-6 – wheat grain naturally contaminated by aflatoxicgenic *Aspergillus* spp.

Mycotoxin detection in stored wheat grain

The results of the multiplex PCR assays were found to be in good agreement with those obtained by chemical analyses of the samples (Table 3), which were subjected for mycotoxin screening by LC-MS/MS. The LOD values obtained in the current study ranged from 0.125 ppb for aflatoxins to 1 ppb for DON. Overall, 17 wheat samples were positive for mycotoxins above their LOD; six samples analysed (~20%) were contaminated with at least one mycotoxin over the EU regulatory limits. AFB_1 was detected in four postharvest wheat samples at levels above the EU regulatory limit of 2 ppb in grains for human consumption (Table 3). The samples were found to be contaminated with *A. flavus* possessing aflatoxin biosynthetic genes (Figure 2A) and *A. flavus*-specific aspergillopepsin *pepO* gene (data not shown). Molecular analyses of the grain samples revealed amplification of the *ver1* and/or *aflR1* genes (Figure 3B), which are associated with aflatoxin biosynthesis. A PCR-based assay of *Fusarium* mycotoxin biosynthetic genes showed amplification of *fum1* and *fum13* genes associated with fumonisin biosynthesis in four wheat samples (Figure 3A, lanes 2-5; samples #18-21). Among the strains isolated from the samples *F. verticillioides* was identified as predominant species, based on morphological and molecular characterization, harbouring the *fum1* and *fum13* genes (Figure 2B). The LC-MS/MS analysis clearly indicated the presence of FB_1 and FB_2 toxins in the samples with high FB_1 contamination (2.34 ppm) in stored grain sample #18 (Table 3). Another two wheat DNA samples (#22, 24) showed amplification of *tri6* gene involved in trichothecene biosynthesis, according to the results of the multiplex PCR assay (Figure 3A, lanes 6, 8). When these DNA samples tested against *Fusarium* species-specific sets of primers the 570 bp amplification fragment was obtained, which indicated the presence of *F.*

culmorum. The grains samples were cultured to confirm identification and found to be contaminated predominantly by *F. culmorum* with DON-producing abilities. Type B trichothecene DON, also known as vomitoxin, has been detected and quantified by LC-MS/MS in both samples, with the concentration beyond the regulatory limits in sample #24 (1.74 ppm; Table 3). The similar strong correlation was found between LC-MS/MS analysis of three stored grain samples (#4-6), with FB_1/FB_2 being present at values below regulatory guidelines, and an established multiplex PCR assay on DNAs extracted from the same wheat samples, resulted in specific amplification of target genes *fum1* and *fum13*. Some differences were, however, observed between molecular and analytical chemistry results. In particular, samples #18-21, in addition to *fum1* and *fum13* fragments, showed amplification of *tri6* gene using multiplex PCR (Figure 3A), whereas the production of only fumonisins was observed by multi-mycotoxin LC-MS analysis. This could be due to absence of proper environmental conditions that might have inhibited the expression of specific toxin metabolic pathway genes (12).

Table 3. Mycotoxin contamination in stored wheat grain samples (ppb)

Sample #	AFB_1	AFB_2	AFG_1	AFG_2	FB_1	FB_2	DON	OTA	T-2	ZEA
1-3	-	-	-	-	-	-	-	-	-	-
4	2.1	-	-	-	179.4	13.3	16.7	-	-	-
5	-	-	-	-	6.2	2.2	-	-	-	-
6	-	-	-	-	428.4	24.5	1.2	-	-	0.7
7	-	-	-	-	4.3	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	0.2	-	-	2.8	-	-	-
10	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	4.6	-	-	-
12-17	-	-	-	-	-	-	-	-	-	-
18	-	-	-	1.1	2340.7	87.9	-	-	-	-
19	-	-	-	0.3	53.9	4	-	-	-	-
20	6.2	-	-	0.4	62.6	4.8	-	-	-	8.9
21	5.2	-	-	0.6	135.1	11.8	-	1.9	-	3.8
22	-	-	-	-	-	-	263.5	-	-	7.6
23	-	-	-	-	-	-		-	-	-
24	-	-	-	-	-	-	1746.5	-	-	64.8
25	-	-	-	-	-	-	-	-	-	-
26	32.3	1.8	-	-	106.7	7.5	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	8.1		-	-
29	-	-	-	-	-	-	-	22.8	-	-
30	-	-	-	-	-	-	-	41.5	-	-
31	-	-	-	-	-	-	5.7	-	-	-
32-34	-	-	-	-	-	-	-	-	-	-

*** – not detected**

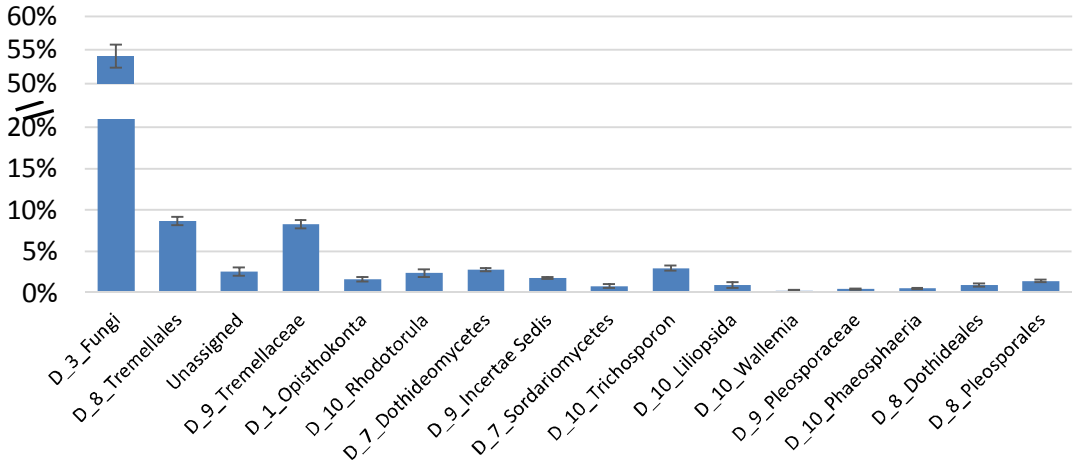
Microbiome composition

The 18S rRNA and 16S rRNA gene sequencing of all stored wheat grain samples generated high quality sequences. Among eukaryotic epiphytes fungal community was the most abundant, followed by Tremellales (another order of fungi in the class Tremellomycetes; Figure 4A). Bacillus was the most predominant bacterial taxa, followed by Erwinia, Pseudomonas and Oxalobacteraceae (Figure 4B). Within-sample

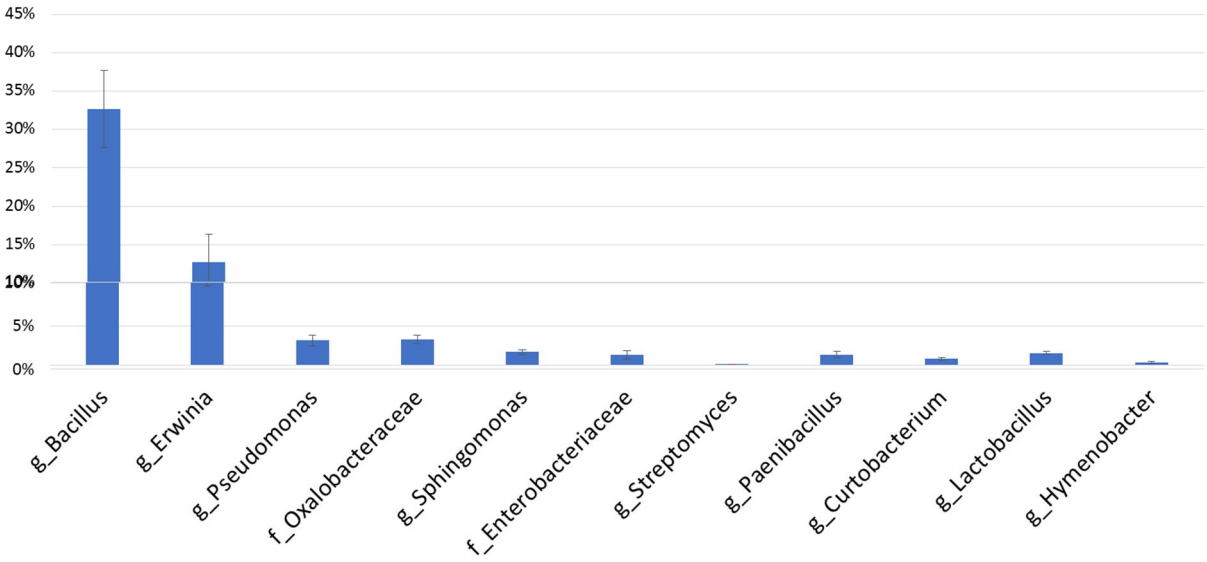
alpha diversity and between-sample beta diversity statistics are currently under process and the results will be submitted in the next continuation project report (due to the limitations of the duration of the current project – 2 years).

Figure 4. Bar charts showing the relative abundance of most dominant taxa in wheat samples

(A) Eukaryotic epiphytes



(B) Bacterial epiphytes



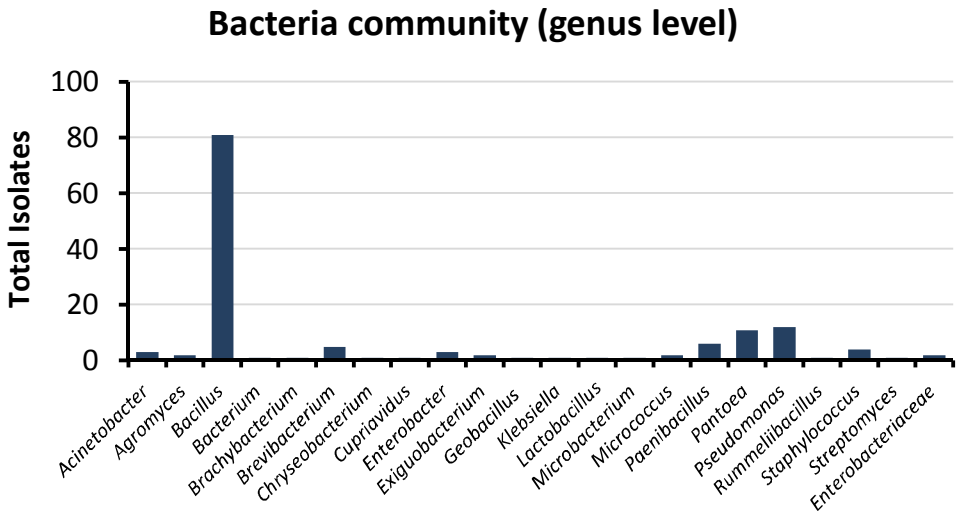
Microbe isolations from stored wheat grains and in vitro assays

In order to assess potential interactions between members of the shared microbiota, we undertook efforts to culture bacterial and fungal isolates. Multiple bacterial colony morphologies were observed on LB agar plates when isolated from wheat grains (see "Materials and methods"). Determination of the amplicon sequences using 16S rRNA universal primers revealed that among bacteria *Bacillus* was prevalent genus isolated (Figure 5A). Fungi were also isolated from wheat grains, with a wide range of colony

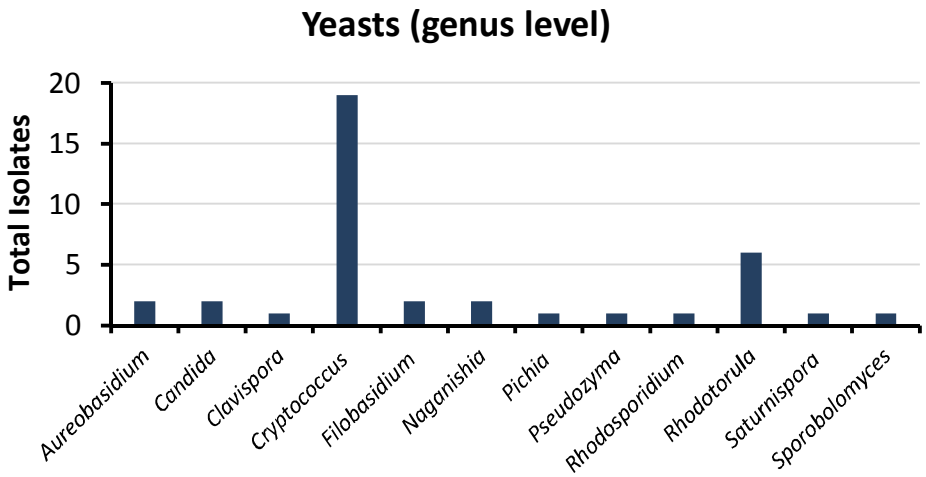
morphologies observed, including yeasts and molds. *Cryptococcus* spp. were predominanat among yeast population isolated from stored whet grains (Figure 5B). *Alternaria* spp. was found in nearly all samples; *Fusarium* and *Aspergillus* spp. were the next most abundant across multiple samples (Figure 5C).

Figure 5. Microbe isolates recovered from stored wheat grains

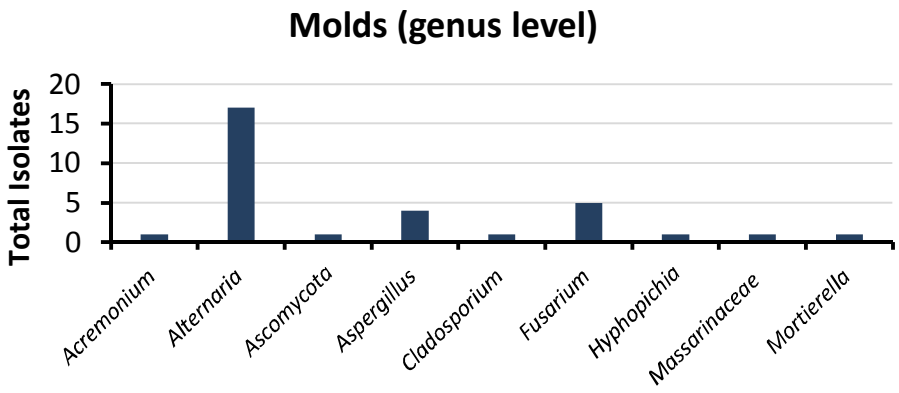
(A) Bacterial isolates



(B) Yeasts isolates

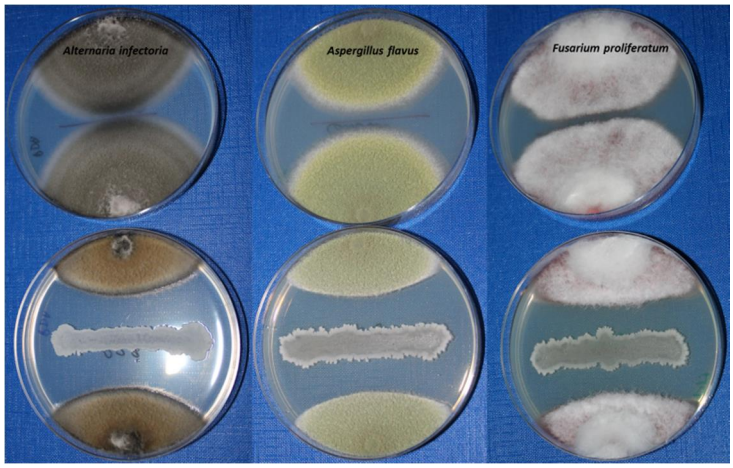


(C) Molds population



Four *Bacillus* spp. from wheat seeds showed a spectrum of growth suppression against fungal isolates. Figure 6 shows strong inhibitory effect of *B. amyloliquefaciens* against the major mycotoxigenic fungi such as *A. flavus*, *Alternaria infectoria* and *F. proliferatum*.

Figure 6. Interactions between selected bacterial isolate and mycotoxigenic fungi



Discussion

The specificity of the developed multiplex PCR assay was achieved since it provided a good discrimination between the tested species as well as mycotoxin producing and non-producing strains. In addition, none of the primers showed cross-reactions with those species amplified by the respective other primers. In the present study the new developed assays have advantages over other previously reported multiplex PCRs in terms of broad range of mycotoxigenic fungi detection in a few single assays, which could detect all target mycotoxigenic fungal species with higher specificity. The sensitivity of PCR reaction might be influenced by various components of biological matrices, such as fats, phenolic and polysaccharide compounds, which can reduce the purity of the extracted DNA (13-15). Yet, in this study, the higher efficacy of the assay

has been achieved due to an efficient DNA extraction method and optimized PCR conditions, and resulted in an improvement of at least one log of sensitivity, compared with that reported in previous studies (13,16). PCR assay may also yield false positive results due to difficulties in detecting mutation outside the primers' targeted region of gene sequence (17). Nevertheless, some research groups pointed out that false positive results in the detection of mycotoxigenic fungi are more acceptable than false negatives for diagnostic purposes of screening food and feed samples (13,18).

It has been previously reported that *F. verticillioides* and *F. culmorum* were isolated from different corn growing areas in Israel (19,20). Due to the rise in average temperatures caused by climate change these species are now frequently reported as the main agents of *Fusarium* diseases of cereals in the Mediterranean region, and particularly in years characterized by wet conditions (21-24). Moreover, *F. verticillioides* and *F. culmorum* are also known as post-harvest pathogens, especially on freshly harvested grain that has not been dried or stored properly (2,25).

A number of stored wheat grain samples were found to be contaminated mainly by *Fusarium* and *Aspergillus* spp. Co-occurrence of different mycotoxins produced by these fungi has been observed: the chemical analysis revealed that 50% of the samples contained mycotoxins above the level of detection. Nearly 20% of the samples were found to be contaminated by at least one mycotoxin over the EU regulatory limits. The strong correlation between multiplex PCR and LC-MS/MS results obtained in the present study provides a rapid, accurate and sensitive detection of mycotoxigenic species and mycotoxins in wheat grain. Combination of molecular and analytical based procedures might serve as a reliable diagnostic tool in food safety laboratories for the rapid screening of large numbers of samples. In addition, such approach can be very useful in obtaining information about the potential toxigenicity of fungal species and their concomitant mycotoxins that might contaminate wide range of agricultural and food products.

The majority of bacterial taxa that we observed in the stored wheat grain microbiome included OTU that were related to *Bacillus* (Fig. 4B). Many of the bacterial microorganisms we identified on the seed surface are *Bacillus* spp., suggesting a high correlation between microbiome analysis and isolation of seed-borne microorganisms. Among the fungal OTU were several with similarity to yeasts and Ascomycetes, including *Fusarium*, *Aspergillus* and *Alternaria*. Sequences identical to known wheat pathogens that can cause grain spoilage were detected on these seeds; given the

ability for the seeds to be associated with microbes closely related to pathogens there is a clear need to monitor seed health. These findings demonstrate that the seed microbiome is crucial as it may harbor both beneficial and potentially pathogenic organisms. *B. amyloliquefaciens* strain we identified in this study has potential as a biocontrol agent, and if applied to seeds may act to protect them from storage-associated spoilage or colonization with pathogenic microorganisms. Our ability to perform high-throughtput sequencing of the stored wheat grain epiphytic microbiome has revealed large numbers of microbes forming communities that can affect disease outcomes. These results provide a system for understanding the microorganisms that are associated with seeds, and highlight the need for a thorough understanding of these microbial communities and their importance to production and storage of healthy, high quality wheat grains.

Publications resulting from the research work

Sadhasivam, S., Britzi, M., Zakin, V., Kostyukovsky, M., Trostanetsky, A., Quinn, E., Sionov, E. (2017). Rapid detection and identification of mycotoxigenic fungi and mycotoxins in stored wheat grain. *Toxins.* 9: 302; doi: 10.3390/toxins9100302.

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Link for the article: http://www.mdpi.com/2072-6651/9/10/302

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