Efficacy of Bacillus subtilis and Bacillus amyloliquefaciens in the control of Aspergillus parasiticus growth and aflatoxins production on pistachio

Efficacy of *Bacillus subtilis* and *Bacillus amyloliquefaciens* in the control of *Aspergillus parasiticus* growth and aflatoxins production on pistachio as a food model system

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Abstract

Pistachio (*Pistacia vera)* is an important nut for its economic, nutritional and health aspects but it can be contaminated by aflatoxigenic fungi in the field and during storage. Biological control could be considered as an alternative to chemical treatment. In this study, we evaluated the antifungal and anti-mycotoxigenic capability of two *Bacillus* spp. both *in vitro* and on pistachio kernels. In *in vitro* conditions, both strains were able to reduce the mycelial growth and they were able to degrade the four aflatoxins during the first three days after inoculation. AFG_1 and AFG_2 were rapidly degraded within two days of incubation with the bacterial strains. No AFs were found in the bacterial cell walls, permitting to exclude the mycotoxin adsorption and to hypothesize an *in vitro* biodegradation. The cultivar of pistachio most susceptible to fungal colonization was 'Ahmad-Aghaei', selected among four main Iranian cultivars. *A. parasiticus* was able to grow and produce aflatoxins on pistachios, but at longer inoculation periods, a natural decrease of aflatoxins was registered. Both strains were able to reduce the fungal incidence and number of spores on pistachio with a stronger effect during the first 5 dpi. The effect on aflatoxin content *in vivo* was less pronounced than *in vitro*, with a maximum effect at 8 dpi. At longer times, there was a contrasting effect due to the lower activity of *Bacillu*s spp. in stationary phase and higher growth of *Aspergillus* species. This consideration could explain the lack of aflatoxin reduction at 12 dpi. Both bacterial strains showed good antifungal activity and aflatoxin reduction in *in vitro* conditions and on pistachio kernels. Altogether, these results indicate that *Bacillus* species could be considered as potential biocontrol agents to combat toxigenic fungal growth and subsequent aflatoxin contamination of nuts and agricultural crops in practice.

Keywords: Aflatoxins, biological control, *Bacillus* species, mycotoxins, *Pistacia vera*, post-harvest management

1. Introduction

Pistachio (*Pistacia vera)* is an important nut for its economic, nutritional and health aspects. This product is original from Middle East. Iran (472,000 tons/year), the United States (231,000), Turkey (150,000) and China (74,000) are the biggest producers of this nut (FAOSTAT, 2014). Iran earns significant income from pistachio export, especially to the European Union.

However, pistachios are susceptible to field fungi that are responsible for the production aflatoxins (AFs), mycotoxins, which limit pistachio export with severe economic losses (Cheraghali et al., 2007). The field fungi, which can produce AFs, belong to the genus *Aspergillus* section *Flavi*. Contamination of nuts with *A. flavus* and *A. parasiticus* occurs universally. While *A. flavus* is more common in agricultural field and is able to produce aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), *A. parasiticus* could produce four AFs, including AFB_1, AFB_2, aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2). AFs are genotoxic and carcinogenic and can cause both acute and chronic toxicity in humans (Williams et al., 2004). AFs were classified by the International Agency for Research on Cancer (IARC, 2004) as carcinogenic agents to humans and animals. The serious health and economic consequences of aflatoxin contamination have created the need for legislative limits, rapid detection techniques, and detoxification strategies (Ricciardi et al., 2013). In Europe, maximum levels in foodstuffs for AFB_1, aflatoxin M_1, and for the sum of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) in nuts are specified by the Commission Regulation (EU) No 165/2010.

Several strategies have been applied to inactivate and detoxify AFs in crops and during postharvest (Spadaro and Garibaldi, 2017), including physical, chemical and biological tools (Siciliano et al., 2016). To avoid fungicide overuse, there is a growing interest in finding alternative methods to chemical control against pathogenic and mycotoxigenic fungi, such as biocontrol agents (BCAs). Applying microorganisms as biological control agents has been increasing in recent decades. The value of the global biopesticide market is expected to reach $4,556.37 million by 2019, at an annual growth rate of 15.3% from 2014 to 2019 (source: Marketsandmarkets.com, 2014).

Several antagonists were applied for this aim, but atoxigenic fungi and bacterial strains are commonly used. The ideal antagonist should have some characteristics, such as genetic stability, efficacy at low concentrations against a wide range of pathogens on fruit products, simple nutritional requirements, survival in adverse environmental conditions, growth on cheap substrates in fermenters, lack of pathogenicity for the host plant and lack of toxigenicity to humans, resistance to the most frequently used pesticides and compatibility with other chemical and physical treatments (Spadaro and Gullino, 2004).

Terrestrial bacteria are an interesting group of antagonistic microorganisms capable of efficiently inhibit toxigenic fungus growth and AF production (Ahmed et al., 2014; Zhao et al, 2012). Many bacterial strains, especially from the genus *Bacillus*, *Pseudomonas*, *Agrobacterium* and *Streptomyces* possess these features (Holmes et al., 2008; Ongena and Jacques, 2007; Ranjbariyan et al., 2011; Razzaghi-Abyaneh et al., 2011; Stein, 2005). Metabolites from *Bacillus subtilis* (Fengycins A and B, plipastatins A and B, iturin A, mycosubtilin, bacillomycin D), *Streptomyces* spp. (dioctatin A, aflastatin A, blasticidin A), and *Achromobacter xylosoxidans*

88 [cyclo (L-leucyl-L-propyl)] are good examples of potent inhibitors of AF biosynthesis in
89 laboratory conditions, crop model systems and in the field (Razzaghi-Abyaneh et al., 2011).
90 Previous studies focused on the inhibitory effect of bacterial strains in *in vitro* experiments or in
91 the control of *A. flavus* on pistachio (Afsharmanesh et al., 2014; Farzaneh et al., 2012). In this
92 study, two bacterial strains, isolated from pistachio orchard soil, were chosen to determine their
93 antifungal and anti-aflatoxigenic activity against *A. parasiticus in vitro* and on an Iranian cultivar
94 of pistachio. Four important Iranian pistachio cultivars were examined to choose the most
95 susceptible one to *A. parasiticus* and to perform biocontrol experiments. The bacterial strains
96 were identified morphologically and molecularly. Two main mechanisms were reported for
97 aflatoxin reduction by antagonistic strains in liquid medium: degradation by enzymes or
98 metabolites and cell wall adsorption, which the latter is the common mechanism of aflatoxin
99 reduction by yeasts and lactic acid bacteria (LAB) (El-Nezami et al., 1998; Pizzolitto et al.,
100 2013). In our experiments, we tried to understand if degradation or cell wall adsorption were
101 involved.
102
103 **2. Materials and methods**
104 *2.1. Pistachio cultivars and microorganisms*
105 The pistachio kernels were obtained at harvest season from the retail stores in Kerman, the major
106 pistachio producing province of Iran. The samples were chosen from four different cultivars
107 including ‘Ahmad-Aghaei’, ‘Akbari’, ‘Kalleh-Ghuhi’ and ‘Owhadi’. They were vacuum-packed
108 and kept in cold conditions until use. Two bacterial strains (UTB2 and UTB3), which were
109 previously isolated from the soil of pistachio orchards and identified morphologically as *Bacillus*
110 spp., were provided by the microbial bank of Pasteur Institute, Tehran, Iran. The freeze-dried

strains were cultured in Brain Heart Infusion broth (BHI broth) medium for 24h in 30 °C at 150 rpm. *Aspergillus parasiticus* NRRL 2999 (ARS Culture Collection, the United States) spores were prepared by adding Tween suspension (0.1%) to fungal tube cultured for 7 days in Sabouraud Dextrose Agar (SDA). The number of spores was adjusted by counting under microscope, using Bürker chamber.

2.2. Molecular identification of the antagonistic strains

The strains were identified using 16S rRNA gene sequence analysis. The freeze-dried strains were cultured on BHI for 24 h at 30 °C and then streaked on Trypticase Soy Agar (TSA) plates. Single colonies were picked and total DNA was extracted using a DNeasy Mini kit (Qiagen) following the manufacturer's instruction. Polymerase chain reaction (PCR) was carried out using the primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3') (Srivastava *et al*., 2008). PCR conditions were as follows: an initial step at 95 °C for 5 min, and 35 cycles at 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1min, followed by a final extension step at 72 °C for 5 min (Srivastava et al., 2008). The PCR products were subjected to electrophoresis in 1% agarose gel stained with safe DNA gel stain (Invitrogen, USA) to confirm the amplification of the DNA. Purification for subsequent analyzing of sequences was done by PCR purification kits (QIAquick, Germany). The results of sequencing were compared with the genomic sequences in NCBI with the BLAST software.

2.3. Antagonistic assay of the bacterial strains against Aspergillus parasiticus

Ten µl of fungal suspension at the concentration of 10^5 spores/ml and 6 µl of the overnight bacterial culture in LB medium were deposited on PDA plate as a spot at three centimeter

distance from each other. The plates were incubated at 28 °C and the radial growth of fungal colony was measured daily for 6 days.

2.4. Aflatoxin reduction in liquid medium

Aflatoxin assay in liquid medium was studied according to Farzaneh et al. (2012) with some modifications. Briefly, the strain was cultured in LB medium for 24 h at 30 ºC in shaking conditions. Two hundred microliters of each bacterial inoculum suspension were added to five ml of LB medium, which was previously contaminated with 50 µg/l of each AFs. Sterile LB medium and LB containing 200 µg/l AFs (50 µg/l of each AF) were used as controls of the experiment. The samples were incubated at 30 ºC for 0 to 120 h, and collected every 24 hours. Each sample was then centrifuged at 10,000 rpm for 10 min. The supernatant was collected and 5 ml were extracted twice with 10 ml of ethyl acetate. The two organic phases were mixed and evaporated to dryness in a rotavapor and the residue was dissolved in 500 µl of water: acetonitrile for aflatoxins analysis.

To understand the aflatoxin reduction mechanism used by the bacterial strains, the cell walls from the *in vitro* aflatoxin experiment were lysed to track for residual aflatoxin. Briefly, the cell pellets were washed after centrifuge and then sonicated for 30 minutes with saline solution and they were washed by centrifuging at 4000 rpm for 10 min. This step was repeated twice (Patharajan et al., 2011). The pellets were extracted twice with 2 ml of ethyl acetate. The two organic phases were mixed and evaporated to dryness in a vacuum concentrator and the residue was dissolved in 500 µl of water:acetonitrile for aflatoxins analysis.

2.5. Susceptibility of pistachio cultivars to A. parasiticus

157 The most important pistachio cultivars of Iran (Ahmad-Aghaei, Akbari, Kalleh-Ghuhi and
158 owhadi) were chosen to test their susceptibility to *Aspergillus* species. The nuts was surface
159 disinfected by immersion in ethanol and, after ethanol evaporation (Bayman et al., 2002), they
160 were shaken in sterile distilled water for 15 minutes. After air-drying, the nuts were transferred
161 to three section Petri dishes. One nut was fixed into each dish section. Six plates were used for
162 each pistachio cultivar and replicate. Three replicates were considered. A hole (3 mm depth) was
163 made on each of the two sides of pistachio. Each hole was inoculated with 5 µl of *A. parasiticus*
164 suspension (10^5 spores/ml). The plates were incubated in a chamber at 30 °C and 85% relative
165 humidity (RH). The number of holes, which were visibly infected, was counted daily for 7 days.
166 We calculated the number of spores after 5 days post inoculation (dpi). Pistachios were shaken
167 into distilled water with 0.2% Tween 80 (Merck) for 6 hours. Then the spores were counted by
168 Burker chamber under optical microscope and reported as spores/ml.
169
170 *2.6. Growth inhibition on pistachio*
171 The pistachio preparation for *in vivo* antagonistic activity was the same described for the
172 susceptibility experiment. Twelve kernels (10 grams) were used for each treatment, each one
173 with two holes. Each hole was treated with 5 µl of antagonistic bacterial cell suspension (10^9
174 cfu/ml). After air-drying, 5 µl of *A. parasiticus* suspension (10^5 spores/ml) were injected into the
175 holes. *Aspergillus parasiticus* inoculated and non-inoculated controls were included. The kernels
176 were placed at 30 °C and 85% RH. The efficacy of both bacterial strains was evaluated by
177 calculating the incidence of *A. parasiticus* for 8 dpi. Moreover, at 5, 8 and 12 dpi, the pistachios
178 were shaken for 6 hours in distilled water and the spores were counted under microscope with a
179 Burker chamber.

2.7. Aflatoxin reduction on pistachio

The pistachios, prepared and treated as described above, were incubated for 5 and 8 days at 30 °C for the analysis of the four aflatoxins, produced by *A. parasiticus*. Before extraction, samples were dried at 45 °C for 24 h in an oven to stop fungal activity (Atehnkeng et al., 2008). Pistachios were ground and extraction was carried out with 50 ml of acetone/water (70:30) solution. Each sample was maintained under stirring conditions in a rotary shaker for 1 h. Then, extract was filtered through paper filter and evaporated to 500 µl in a rotavapor at 45 °C, the mixture was centrifuged at 6,000 rpm for 5 min and the supernatant was filtered through 0.45 µm filters before HPLC-MS/MS analysis.

2.8. HPLC-MS/MS analysis

Analysis was performed using a Varian Model 212-LC micro pumps (Palo Alto, CA, USA) with a Varian auto sampler Model 410 Prostar coupled with a Varian 310-MS triple quadrupole mass spectrometer with an electrospray ion source (ESI) operating in positive ionization mode. Chromatographic separation was performed in isocratic mode on a Pursuit XRs Ultra C18 (100mm x 2.0 mm, 2.8 µm, Varian) column using as eluents water acidified with 0.05% of formic acid (Sigma-Aldrich, USA) and methanol (Merck, Germany) (40:60 v/v), the flow rate was set at 0.2 ml/min for 10 minutes. Monitoring reaction mode (MRM) transitions used for quantification were: 313>285 (CE 14 V) for AFB_1, 315>287 (CE 18 V) for AFB_2, 329>243 (CE 18 V) for AFG_1, 331>245 (CE 24 V) for AFG_2.

2.9. Statistical analysis

The experiments were performed three times. Data analysis was performed by SPSS software version 23.0. Data were analyzed by one-way analysis of variance (ANOVA) by applying Tukey test. Statistical significance was defined as $P<0.05$.

3. Results and Discussion

3.1. Identification of antagonistic bacteria

Two bacterial strains, isolated from the soil of pistachio orchards, with antagonistic activity against *A. parasiticus,* were identified by 16S ribosomal DNA sequencing. The bacterial sequences were blasted in Genbank and UTB2 was identified as *Bacillus amyloliquefaciens* (accession number: KX587515) and UTB3 as *Bacillus subtilis* (accession number: KX587514).

3.2. Antagonistic assay of the bacterial strains against A. parasiticus

The fungal mycelium and the bacterial colonies were co-cultured in PDA plates for 8 days. Both bacteria showed mycelial growth inhibition on *A. parasiticus* NRRL2999 (Figure 1). Up to three days of co-culture, the radial growth was not different compared to the control. From the 4^{th} day of co-culture, the mycelial growth showed a significant reduction in co-culture compared to the control. The maximum radial growth was reached at 6 days of co-culture (Figure 2).

3.3. Aflatoxin reduction by bacterial strains in liquid medium

Both bacterial strains had similar capability to reduce significantly the aflatoxin content in liquid medium. According to the results shown in Figure 3, the total AFs rapidly decreased during the first three days, while afterwards, there was no more significant reduction. By considering the

single aflatoxins, AFB_1 and AFB_2 seemed more resistant to both antagonistic strains and their residual concentration continued to decrease during the 5 days of the experiment. On the opposite, AFG_1 and AFG_2 were rapidly degraded within two days of incubation with the bacterial strains. Our results are substantially in agreement with other studies, but only the effect on AFB_1 was investigated with higher reduction after 72 hours of incubation (Alberts et al., 2009; Farzaneh et al., 2012; Rao et al., 2016; Teniola et al., 2009). At 72 h incubation, most bacterial cells enter their stationary phase, where more secondary metabolites, including antibiotics and lytic enzymes, are produced (Sansinen & Ortiz, 2011). In addition, reduction of AFs content increased with increasing the bacterial population in the liquid medium (Farzaneh et al., 2012).

The possible mechanisms for aflatoxin reduction in liquid medium were investigated by extracting the AFs also from the cell pellets, from the day 1 to day 5 of incubation. According to HPLC analysis, no AFs were found in the bacterial cell walls. Therefore, the main mechanism for aflatoxin reduction in liquid medium was toxin degradation, which is probably due to bacterial enzymatic activity. It has been proved that *B. subtilis* and *B. amyloliquefaciens* produce laccase enzyme (Alberts et al., 2009), which could be involved in degrading AFs.

3.4. Susceptibility of pistachio cultivars to A. parasiticus

To determine the antagonistic activity of the bacteria against *A. parasiticus* in pistachios, the cultivar of pistachio most susceptible to fungal colonization was selected among the four most important Iranian cultivars. All the pistachio cultivars were susceptible to *A. parasiticus*. Already at 2 dpi, the fungus colonized the kernel surface (Figure 4A). The incidence increased until 5 or 6 dpi, depending on the cultivar. Only the cultivar Ahmad-Aghaei reached an incidence of 100%

249 at 5 dpi, showing to be more susceptible than the other three to *A. parasiticus*. The cultivars
250 Ahmad-Aghaei and Owhadi showed the highest number of fungal spores with no significant
251 difference ($p < 0.05$), while the cultivar Kalleh-Ghuchi showed the lowest number of spores
252 (Figure 4B). Moghadam and Hokmabadi (2010) studied the susceptibility of some Iranian
253 cultivars to a toxigenic *A. flavus*, which belong to the same section *Flavi* of *Aspergillus* as *A.*
254 *parasiticus*. Similarly, the most susceptible cultivar to colonization of *A. flavus* was 'Ahmad-
255 Aghaei'. As the most susceptible cultivar to *A. parasiticus* was 'Ahmad-Aghaei', it was chosen
256 for the biocontrol experiments.
257
258 *3.5. Aflatoxin production of* A. parasiticus *on pistachio 'Ahmad-Aghaei'*
259 The AFs production of *A. parasiticus* on pistachio kernels was measured at 5, 8 and 12 dpi.
260 Three replicates of pistachios without fungal inoculation were extracted for initial aflatoxin
261 content. No measurable AFs were recorded for the controls. The highest amount of AFs was
262 recorded at 5 dpi (526 ng/g pistachio). AFG_1 and AFG_2 were more produced than AFB_1 and
263 AFB_2, respectively. AFs content on inoculated pistachios declined during the experiment. A
264 significant decrease was observed at 12 dpi. The aflatoxin content of *Aspergillus* belonging to
265 the section *Flavi* decreased at 8 dpi in other experiments (Alberts et al., 2009; Bin-Hamid and
266 Smith, 1987; Shih and Marth., 1975). In raisins inoculated with *A. parasiticus* and incubated for
267 15 days at 30 °C (Kostarelou et al., 2014), the aflatoxin content reached the peak at the 9^{th} day,
268 and later started to decrease. *A. parasiticus* and *A. flavus* produce AFs for a limited period,
269 depending on nutritional sources and environmental factors such as humidity. After that,
270 aflatoxins are reabsorbed and/or metabolized (Bin-Hamid and Smith, 1987). For this reason, the

following experiments were carried out for 5 and 8 dpi, as at 12 days there was a natural aflatoxin reduction.

3.6. Efficacy of bacterial strains against A. parasiticus *in pistachio*

Co-inoculation of bacterial strains with *A. parasiticus* reduced the fungal colonization on pistachio kernels (Figure 5A). The bacterial strains were able to reduce the incidence of *A. parasiticus* until 5 dpi. Afterwards, the incidence of the treated kernels was similar to the control. The samples treated with antagonists contained lower amount of spores during the 12 days of incubation in comparison to the control. *B. subtilis* showed a slightly higher reduction of the number of fungal spores. More than two logs reduction were recorded for *B. subtilis* at 5 and 8 dpi, while around 1.5 logs reduction at 12 dpi, in comparison to control (Figure 5B). Production of antifungal metabolites and competition for space and nutrition seem to be the possible mechanisms of antagonistic activity of bacterial strains *in vivo* (Kong et al., 2010; Kumar et al., 2014).

3.7. Efficacy of bacterial strains on aflatoxin reduction in pistachio

Pistachios inoculated with *A. parasiticus* and treated with bacteria showed a lower AFs content in comparison to the untreated control. The highest reduction for AFB_1 was recorded at 8 dpi for *B. amyloliquefaciens* (54.9%) and *B. subtilis* (52.5%). For total aflatoxins, the highest reduction was registered at 8 dpi for UTB2 (52.4%) and UTB3 (45.9%). At 12 dpi (data not shown), the treatment with either antagonist was unable to significantly reduce the aflatoxin content compared to the control.

As strains of *Bacillus* spp. are growing at a faster rate than *Aspergillus* spp., they showed a higher biocontrol efficacy during the first steps of co-incubation, reaching the maximum effect at 5 dpi. Later, the bacterial strains entered a stationary phase, characterized by endospore production, while the fungus continued to grow (Pepe et al., 2013). This consideration could explain the lack of aflatoxin reduction at 12 dpi.

4. Conclusions

The pistachio can be contaminated by aflatoxins in the field and during storage. Therefore, a control strategy of aflatoxigenic fungi is necessary. Biological control could be considered an alternative to pesticide treatment. In this study, we evaluated the antifungal and anti-mycotoxigenic capability of two field isolates of *Bacillus* spp. both *in vitro* and on pistachio kernels. In vitro both strains were able to reduce the mycelial growth and they were able to degrade the four aflatoxins during the first three days. AFG_1 and AFG_2 were rapidly degraded within two days of incubation with the bacterial strains. No AFs were found in the bacterial cell walls, permitting to exclude the mycotoxin adsorption and to hypothesize an *in vitro* biodegradation. The cultivar of pistachio most susceptible to fungal colonization was 'Ahmad-Aghaei', selected among four main Iranian cultivars. *A. parasiticus* was able to grow and produce aflatoxins on pistachios, but at longer inoculation periods, a natural decrease of aflatoxins was registered. Both strains were able to reduce the fungal incidence and number of spores on pistachio with a stronger effect during the first 5 dpi. The effect on aflatoxin content *in vivo* was less pronounced than *in vitro*, with a maximum effect at 8 dpi. At longer times, there was a contrasting effect due to the lower activity of *Bacillu*s spp. in stationary phase and higher growth of *Aspergillus* spp.. This consideration could explain the lack of aflatoxin reduction at 12

316 dpi. Both bacterial strains showed good antifungal activity and aflatoxin reduction in *in vitro*
317 conditions and on pistachio kernels but further research is required to formulate the biocontrol
318 agents in order to survive for longer times.
319
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323
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Table 1 - Reduction of aflatoxins AFB_1, AFB_2, AFG_1, and AFG_2 at 5 time points (24 to 120 h) due to culture in PDB medium at 30 °C with bacterial strains UTB2 and UTB3.

Hours	Bacterial strain	Aflatoxin reduction (%)*				
		AFB_1	AFB_2	AFG_1	AFG_2	AFs
24	*UTB2*	43.2^{d}	33.3^{e}	49.7^{d}	24.1^{c}	37.6
48	*UTB2*	55.2^{c}	41.4^{d}	73.7^{c}	88.5^{b}	64.7
72	*UTB2*	64.6^{b}	51.3^{bc}	94.7^{b}	99.7^{a}	78.8
96	*UTB2*	68.4^{ab}	54.0^{b}	94.1^{b}	99.5^{a}	79.0
120	*UTB2*	70.9^{a}	53.1^{b}	96.8^{a}	99.8^{a}	80.0
24	*UTB3*	37.1^{e}	28.1^{e}	33.6^{e}	17.9^{d}	27.8
48	*UTB3*	50.7^{c}	45.3^{cd}	75.1^{c}	99.0^{a}	67.5
72	*UTB3*	70.2^{a}	61.8^{a}	94.3^{a}	99.7^{a}	82.7
96	*UTB3*	70.2^{a}	62.2^{a}	99.8^{a}	99.8^{a}	83.0
120	*UTB3*	72.6^{a}	64.6^{a}	99.8^{a}	99.8^{a}	84.2

*Values in the same column followed by the same letter are not statistically different by Tukey Test ($P < 0.05$).

Table 2 - Aflatoxin production of *A. parasiticus* strain NRRL 2999 on kernels of pistachio 'Ahmad-Aghaei' after 5 days of inoculation at 30 °C and 85% relative humidity.

Days post inoculation	AFs (ng/g pistachio)*				
	AFB_1	AFB_2	AFG_1	AFG_2	AFs
0	nd	nd	nd	nd	nd
5	176[a]	14.3[a]	312[a]	24.0[a]	526[a]
8	170[a]	14.0[a]	309[a]	22.0[a]	505[a]
12	88.3[b]	6.9[b]	60.6[b]	3.4[b]	159[b]

nd: not detected

*Values in the same column followed by the same letter are not statistically different by Tukey Test ($P < 0.05$).

Table 3 - Aflatoxin reduction induced by bacterial strains UTB2 and UTB3 grown on pistachio 'Ahmad-Aghaei' inoculated with *A. parasiticus* strain NRRL 2999 for 5 and 8 days at 30 °C.

Days	Bacterial strain	Aflatoxin reduction (%)*				
		AFB_1	AFB_2	AFG_1	AFG_2	AFs
5	*UTB2*	23.9^{b}	33.3^{c}	41.0^{b}	49.9^{a}	37.0
8	*UTB2*	54.9^{a}	64.3^{a}	39.5^{b}	50.9^{a}	52.4
5	*UTB3*	$41.1a^{b}$	36.5^{c}	49.0^{a}	42.2^{b}	42.2
8	*UTB3*	52.5^{a}	46.6^{b}	39.2^{b}	45.2^{b}	45.9

*Values in the same column followed by the same letter are not statistically different by Tukey Test ($P < 0.05$).

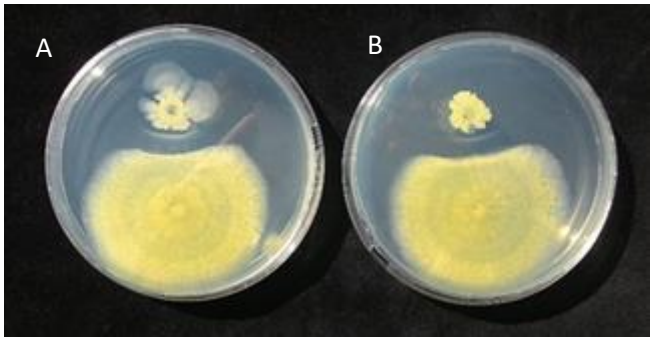

Fig. 1 - Mycelial inhibition of *A. parasiticus* NRRL 2999 grown in the presence of bacterial strains in PDA medium at 30 °C for 6 days. A) *A. parasiticus* co-cultured with *B. subtilis* strain UTB2, *B) A. parasiticus* co-cultured with *B. amyloliquefaciens* UTB3.

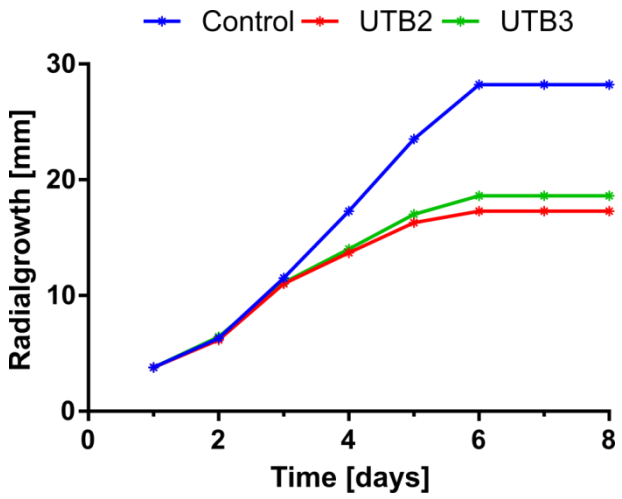

Fig. 2 - Mycelial radial growth of *A. parasiticus* strain NRRL 2999 grown in PDA at 30°C for 8 days alone (A) or in presence of strain UTB2 (B) or UTB3 (C).

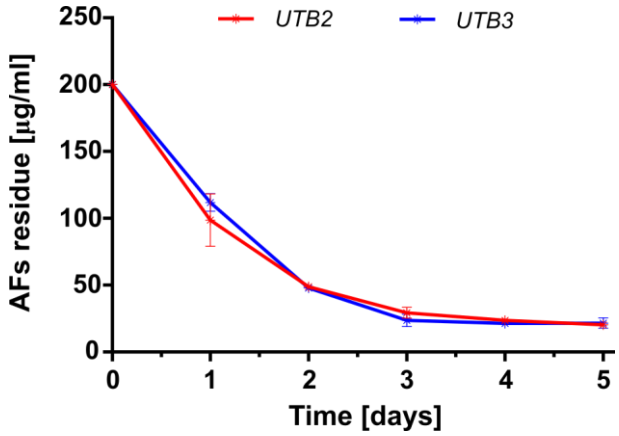

Fig. 3 - Total aflatoxins at 6 time points (from 0 to 5 days) due to culture in PDB medium at 30 °C with bacterial strains UTB2 and UTB3. Initial concentration of total AFs was 200 ppb. Error bars are calculated on three biological replicates.

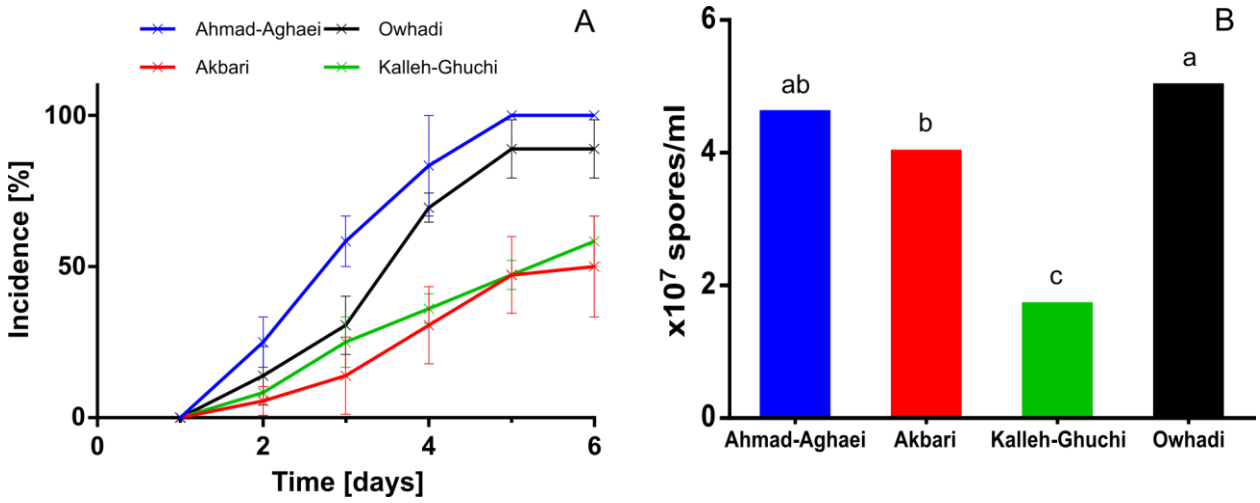

Fig. 4 - Susceptibility of pistachio 'Ahmad-Aghaei' kernels to *A. parasiticus* mould after inoculation at 30 °C and 85% relative humidity. Incidence (%) of infected wounds from 0 to 7 days (A). Number of spores/ml produced on the surface of pistachio kernels at 5 days of inoculation (B).

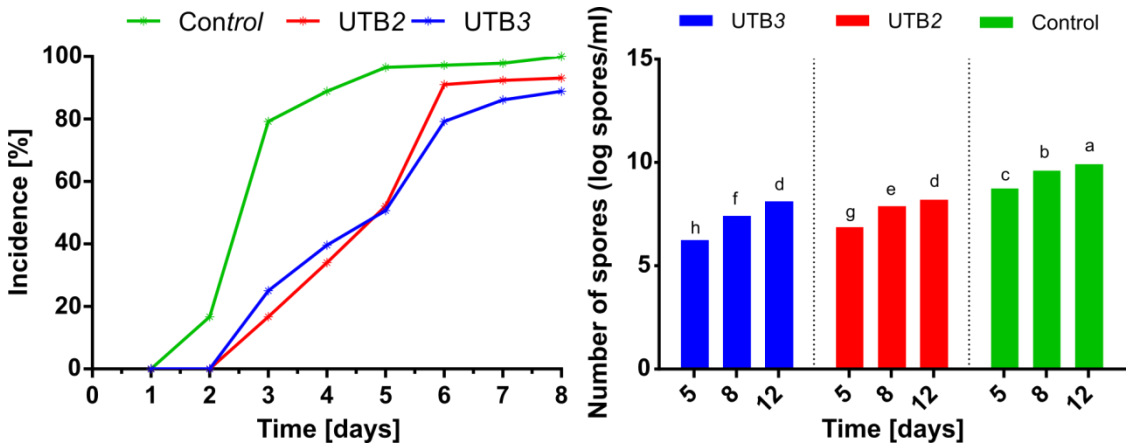

Fig. 5 - Growth of *A. parasiticus* strain NRRL 2999 in presence of strain UTB2 (red) or UTB3 (blue) or alone (green) on kernels of pistachio 'Ahmad-Aghaei' after inoculation at 30 °C and 85% relative humidity. Incidence (%) of infected wounds from 0 to 8 days (A). Log (number of spores/ml) produced on the surface of pistachio kernels at 5, 8 and 12 days of inoculation (B).