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

4
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20

21 **Abstract**

22 Pistachio (*Pistacia vera*) is an important nut for its economic, nutritional and health aspects but it
23 can be contaminated by aflatoxigenic fungi in the field and during storage. Biological control
24 could be considered as an alternative to chemical treatment. In this study, we evaluated the
25 antifungal and anti-mycotoxigenic capability of two *Bacillus* spp. both *in vitro* and on pistachio
26 kernels. In *in vitro* conditions, both strains were able to reduce the mycelial growth and they
27 were able to degrade the four aflatoxins during the first three days after inoculation. AFG₁ and
28 AFG₂ were rapidly degraded within two days of incubation with the bacterial strains. No AFs
29 were found in the bacterial cell walls, permitting to exclude the mycotoxin adsorption and to
30 hypothesize an *in vitro* biodegradation. The cultivar of pistachio most susceptible to fungal
31 colonization was ‘Ahmad-Aghaei’, selected among four main Iranian cultivars. *A. parasiticus*
32 was able to grow and produce aflatoxins on pistachios, but at longer inoculation periods, a
33 natural decrease of aflatoxins was registered. Both strains were able to reduce the fungal
34 incidence and number of spores on pistachio with a stronger effect during the first 5 dpi. The
35 effect on aflatoxin content *in vivo* was less pronounced than *in vitro*, with a maximum effect at 8
36 dpi. At longer times, there was a contrasting effect due to the lower activity of *Bacillus* spp. in
37 stationary phase and higher growth of *Aspergillus* species. This consideration could explain the
38 lack of aflatoxin reduction at 12 dpi. Both bacterial strains showed good antifungal activity and
39 aflatoxin reduction in *in vitro* conditions and on pistachio kernels. Altogether, these results
40 indicate that *Bacillus* species could be considered as potential biocontrol agents to combat
41 toxigenic fungal growth and subsequent aflatoxin contamination of nuts and agricultural crops in
42 practice.

43

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

46

47 **1. Introduction**

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

52 However, pistachios are susceptible to field fungi that are responsible for the production
53 aflatoxins (AFs), mycotoxins, which limit pistachio export with severe economic losses
54 (Cheraghali et al., 2007). The field fungi, which can produce AFs, belong to the genus
55 *Aspergillus* section *Flavi*. Contamination of nuts with *A. flavus* and *A. parasiticus* occurs
56 universally. While *A. flavus* is more common in agricultural field and is able to produce aflatoxin
57 B₁ (AFB₁), aflatoxin B₂ (AFB₂), *A. parasiticus* could produce four AFs, including AFB₁, AFB₂,
58 aflatoxin G₁ (AFG₁) and aflatoxin G₂ (AFG₂). AFs are genotoxic and carcinogenic and can cause
59 both acute and chronic toxicity in humans (Williams et al., 2004). AFs were classified by the
60 International Agency for Research on Cancer (IARC, 2004) as carcinogenic agents to humans
61 and animals. The serious health and economic consequences of aflatoxin contamination have
62 created the need for legislative limits, rapid detection techniques, and detoxification strategies
63 (Ricciardi et al., 2013). In Europe, maximum levels in foodstuffs for AFB₁, aflatoxin M₁, and for
64 the sum of aflatoxins (AFB₁, AFB₂, AFG₁ and AFG₂) in nuts are specified by the Commission
65 Regulation (EU) No 165/2010.

66 Several strategies have been applied to inactivate and detoxify AFs in crops and during
67 postharvest (Spadaro and Garibaldi, 2017), including physical, chemical and biological tools
68 (Siciliano et al., 2016). To avoid fungicide overuse, there is a growing interest in finding
69 alternative methods to chemical control against pathogenic and mycotoxigenic fungi, such as
70 biocontrol agents (BCAs). Applying microorganisms as biological control agents has been
71 increasing in recent decades. The value of the global biopesticide market is expected to reach
72 \$4,556.37 million by 2019, at an annual growth rate of 15.3% from 2014 to 2019 (source:
73 Marketsandmarkets.com, 2014).

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

81 Terrestrial bacteria are an interesting group of antagonistic microorganisms capable of efficiently
82 inhibit toxigenic fungus growth and AF production (Ahmed et al., 2014; Zhao et al, 2012). Many
83 bacterial strains, especially from the genus *Bacillus*, *Pseudomonas*, *Agrobacterium* and
84 *Streptomyces* possess these features (Holmes et al., 2008; Ongena and Jacques, 2007;
85 Ranjbariyan et al., 2011; Razzaghi-Abyaneh et al., 2011; Stein, 2005). Metabolites from *Bacillus*
86 *subtilis* (Fengycins A and B, plipastatins A and B, iturin A, mycosubtilin, bacillomycin D),
87 *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

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

116

117 *2.2. Molecular identification of the antagonistic strains*

118 The strains were identified using 16S rRNA gene sequence analysis. The freeze-dried strains
119 were cultured on BHI for 24 h at 30 °C and then streaked on Trypticase Soy Agar (TSA) plates.
120 Single colonies were picked and total DNA was extracted using a DNeasy Mini kit (Qiagen)
121 following the manufacturer's instruction. Polymerase chain reaction (PCR) was carried out using
122 the primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-
123 TACGGCTACCTTGTTACGACTT-3') (Srivastava *et al.*, 2008). PCR conditions were as
124 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
125 °C for 1min, followed by a final extension step at 72 °C for 5 min (Srivastava *et al.*, 2008). The
126 PCR products were subjected to electrophoresis in 1% agarose gel stained with safe DNA gel
127 stain (Invitrogen, USA) to confirm the amplification of the DNA. Purification for subsequent
128 analyzing of sequences was done by PCR purification kits (QIAquick, Germany). The results of
129 sequencing were compared with the genomic sequences in NCBI with the BLAST software.

130

131 *2.3. Antagonistic assay of the bacterial strains against Aspergillus parasiticus*

132 Ten µl of fungal suspension at the concentration of 10⁵ spores/ml and 6 µl of the overnight
133 bacterial culture in LB medium were deposited on PDA plate as a spot at three centimeter

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

136

137 *2.4. Aflatoxin reduction in liquid medium*

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

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

155

156 *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 were 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 μ l 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 μ l of antagonistic bacterial cell suspension (10^9
174 cfu/ml). After air-drying, 5 μ l 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.

180

181 *2.7. Aflatoxin reduction on pistachio*

182 The pistachios, prepared and treated as described above, were incubated for 5 and 8 days at 30
183 °C for the analysis of the four aflatoxins, produced by *A. parasiticus*. Before extraction, samples
184 were dried at 45 °C for 24 h in an oven to stop fungal activity (Atehnkeng et al., 2008).
185 Pistachios were ground and extraction was carried out with 50 ml of acetone/water (70:30)
186 solution. Each sample was maintained under stirring conditions in a rotary shaker for 1 h. Then,
187 extract was filtered through paper filter and evaporated to 500 µl in a rotavapor at 45 °C, the
188 mixture was centrifuged at 6,000 rpm for 5 min and the supernatant was filtered through 0.45 µm
189 filters before HPLC-MS/MS analysis.

190

191 *2.8. HPLC-MS/MS analysis*

192 Analysis was performed using a Varian Model 212-LC micro pumps (Palo Alto, CA, USA) with
193 a Varian auto sampler Model 410 Prostar coupled with a Varian 310-MS triple quadrupole mass
194 spectrometer with an electrospray ion source (ESI) operating in positive ionization mode.
195 Chromatographic separation was performed in isocratic mode on a Pursuit XR_s Ultra C18
196 (100mm x 2.0 mm, 2.8 µm, Varian) column using as eluents water acidified with 0.05% of
197 formic acid (Sigma-Aldrich, USA) and methanol (Merck, Germany) (40:60 v/v), the flow rate
198 was set at 0.2 ml/min for 10 minutes. Monitoring reaction mode (MRM) transitions used for
199 quantification were: 313>285 (CE 14 V) for AFB₁, 315>287 (CE 18 V) for AFB₂, 329>243 (CE
200 18 V) for AFG₁, 331>245 (CE 24 V) for AFG₂.

201

202 *2.9. Statistical analysis*

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

206

207 **3. Results and Discussion**

208

209 *3.1. Identification of antagonistic bacteria*

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

214

215 *3.2. Antagonistic assay of the bacterial strains against A. parasiticus*

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

221

222 *3.3. Aflatoxin reduction by bacterial strains in liquid medium*

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

226 single aflatoxins, AFB₁ and AFB₂ seemed more resistant to both antagonistic strains and their
227 residual concentration continued to decrease during the 5 days of the experiment. On the
228 opposite, AFG₁ and AFG₂ were rapidly degraded within two days of incubation with the
229 bacterial strains. Our results are substantially in agreement with other studies, but only the effect
230 on AFB₁ was investigated with higher reduction after 72 hours of incubation (Alberts et al.,
231 2009; Farzaneh et al., 2012; Rao et al., 2016; Teniola et al., 2009). At 72 h incubation, most
232 bacterial cells enter their stationary phase, where more secondary metabolites, including
233 antibiotics and lytic enzymes, are produced (Sansinen & Ortiz, 2011). In addition, reduction of
234 AFs content increased with increasing the bacterial population in the liquid medium (Farzaneh et
235 al., 2012).

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

242

243 *3.4. Susceptibility of pistachio cultivars to A. parasiticus*

244 To determine the antagonistic activity of the bacteria against *A. parasiticus* in pistachios, the
245 cultivar of pistachio most susceptible to fungal colonization was selected among the four most
246 important Iranian cultivars. All the pistachio cultivars were susceptible to *A. parasiticus*. Already
247 at 2 dpi, the fungus colonized the kernel surface (Figure 4A). The incidence increased until 5 or
248 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). AFG₁ and AFG₂ were more produced than AFB₁ and
263 AFB₂, 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 9th 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

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

273

274 3.6. Efficacy of bacterial strains against *A. parasiticus* in pistachio

275 Co-inoculation of bacterial strains with *A. parasiticus* reduced the fungal colonization on
276 pistachio kernels (Figure 5A). The bacterial strains were able to reduce the incidence of *A.*
277 *parasiticus* until 5 dpi. Afterwards, the incidence of the treated kernels was similar to the control.
278 The samples treated with antagonists contained lower amount of spores during the 12 days of
279 incubation in comparison to the control. *B. subtilis* showed a slightly higher reduction of the
280 number of fungal spores. More than two logs reduction were recorded for *B. subtilis* at 5 and 8
281 dpi, while around 1.5 logs reduction at 12 dpi, in comparison to control (Figure 5B). Production
282 of antifungal metabolites and competition for space and nutrition seem to be the possible
283 mechanisms of antagonistic activity of bacterial strains *in vivo* (Kong et al., 2010; Kumar et al.,
284 2014).

285

286 3.7. Efficacy of bacterial strains on aflatoxin reduction in pistachio

287 Pistachios inoculated with *A. parasiticus* and treated with bacteria showed a lower AFs content
288 in comparison to the untreated control. The highest reduction for AFB₁ was recorded at 8 dpi for
289 *B. amyloliquefaciens* (54.9%) and *B. subtilis* (52.5%). For total aflatoxins, the highest reduction
290 was registered at 8 dpi for UTB2 (52.4%) and UTB3 (45.9%). At 12 dpi (data not shown), the
291 treatment with either antagonist was unable to significantly reduce the aflatoxin content
292 compared to the control.

293 As strains of *Bacillus* spp. are growing at a faster rate than *Aspergillus* spp., they showed a
294 higher biocontrol efficacy during the first steps of co-incubation, reaching the maximum effect at
295 5 dpi. Later, the bacterial strains entered a stationary phase, characterized by endospore
296 production, while the fungus continued to grow (Pepe et al., 2013). This consideration could
297 explain the lack of aflatoxin reduction at 12 dpi.

298

299 **4. Conclusions**

300 The pistachio can be contaminated by aflatoxins in the field and during storage. Therefore, a
301 control strategy of aflatoxigenic fungi is necessary. Biological control could be considered an
302 alternative to pesticide treatment. In this study, we evaluated the antifungal and anti-
303 mycotoxigenic capability of two field isolates of *Bacillus* spp. both *in vitro* and on pistachio
304 kernels. *In vitro* both strains were able to reduce the mycelial growth and they were able to
305 degrade the four aflatoxins during the first three days. AFG₁ and AFG₂ were rapidly degraded
306 within two days of incubation with the bacterial strains. No AFs were found in the bacterial cell
307 walls, permitting to exclude the mycotoxin adsorption and to hypothesize an *in vitro*
308 biodegradation. The cultivar of pistachio most susceptible to fungal colonization was ‘Ahmad-
309 Aghaei’, selected among four main Iranian cultivars. *A. parasiticus* was able to grow and
310 produce aflatoxins on pistachios, but at longer inoculation periods, a natural decrease of
311 aflatoxins was registered. Both strains were able to reduce the fungal incidence and number of
312 spores on pistachio with a stronger effect during the first 5 dpi. The effect on aflatoxin content *in*
313 *vivo* was less pronounced than *in vitro*, with a maximum effect at 8 dpi. At longer times, there
314 was a contrasting effect due to the lower activity of *Bacillus* spp. in stationary phase and higher
315 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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424

425 **Table 1** - Reduction of aflatoxins AFB₁, AFB₂, AFG₁, and AFG₂ at 5 time points (24 to 120 h)
 426 due to culture in PDB medium at 30 °C with bacterial strains UTB2 and UTB3.

427

Hours	Bacterial strain	Aflatoxin reduction (%)*				
		AFB ₁	AFB ₂	AFG ₁	AFG ₂	AFs
24	<i>UTB2</i>	43.2 ^d	33.3 ^e	49.7 ^d	24.1 ^c	37.6
48	<i>UTB2</i>	55.2 ^c	41.4 ^d	73.7 ^c	88.5 ^b	64.7
72	<i>UTB2</i>	64.6 ^b	51.3 ^{bc}	94.7 ^b	99.7 ^a	78.8
96	<i>UTB2</i>	68.4 ^{ab}	54.0 ^b	94.1 ^b	99.5 ^a	79.0
120	<i>UTB2</i>	70.9 ^a	53.1 ^b	96.8 ^a	99.8 ^a	80.0
24	<i>UTB3</i>	37.1 ^e	28.1 ^e	33.6 ^e	17.9 ^d	27.8
48	<i>UTB3</i>	50.7 ^c	45.3 ^{cd}	75.1 ^c	99.0 ^a	67.5
72	<i>UTB3</i>	70.2 ^a	61.8 ^a	94.3 ^a	99.7 ^a	82.7
96	<i>UTB3</i>	70.2 ^a	62.2 ^a	99.8 ^a	99.8 ^a	83.0
120	<i>UTB3</i>	72.6 ^a	64.6 ^a	99.8 ^a	99.8 ^a	84.2

428

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

431

432 **Table 2** - Aflatoxin production of *A. parasiticus* strain NRRL 2999 on kernels of pistachio ‘Ahmad-
 433 Aghaei’ after 5 days of inoculation at 30 °C and 85% relative humidity.
 434

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

435 nd: not detected

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

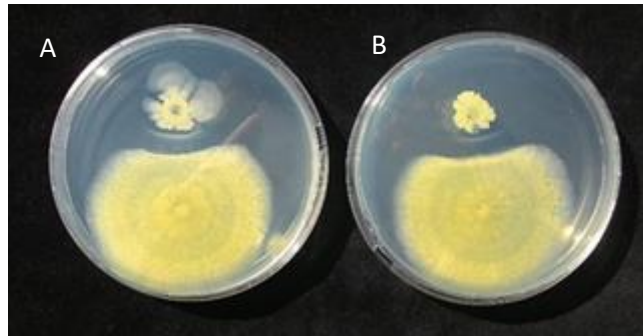
438

439 **Table 3** - Aflatoxin reduction induced by bacterial strains UTB2 and UTB3 grown on pistachio
 440 ‘Ahmad-Aghaei’ inoculated with *A. parasiticus* strain NRRL 2999 for 5 and 8 days at 30 °C.
 441

Days	Bacterial strain	Aflatoxin reduction (%)*				
		AFB ₁	AFB ₂	AFG ₁	AFG ₂	AFs
5	<i>UTB2</i>	23.9 ^b	33.3 ^c	41.0 ^b	49.9 ^a	37.0
8	<i>UTB2</i>	54.9 ^a	64.3 ^a	39.5 ^b	50.9 ^a	52.4
5	<i>UTB3</i>	41.1 ^{a^b}	36.5 ^c	49.0 ^a	42.2 ^b	42.2
8	<i>UTB3</i>	52.5 ^a	46.6 ^b	39.2 ^b	45.2 ^b	45.9

442
 443 *Values in the same column followed by the same letter are not statistically different by Tukey
 444 Test (P < 0.05).

445

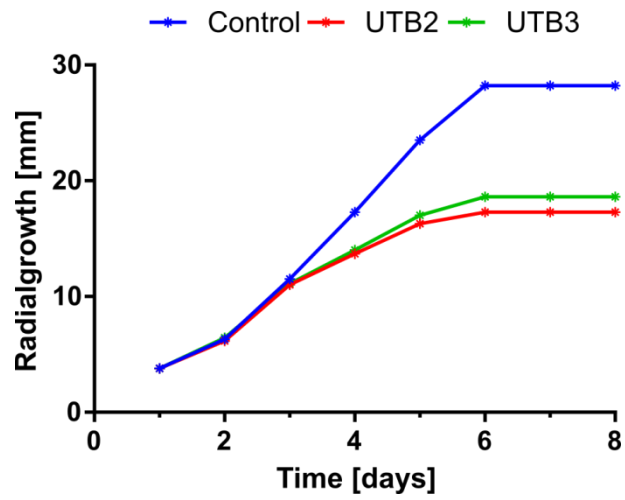


446

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

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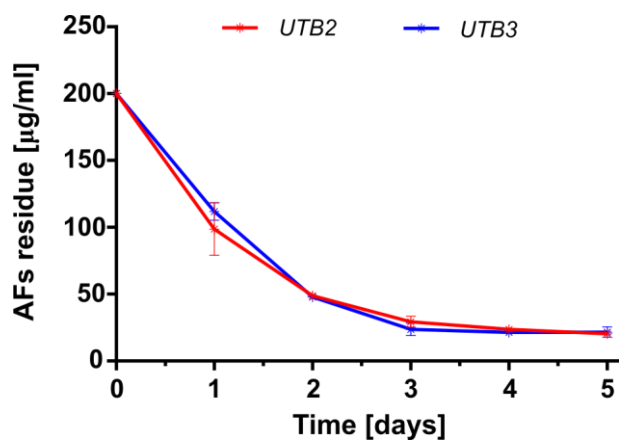
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454 **Fig. 2** - Mycelial radial growth of *A. parasiticus* strain NRRL 2999 grown in PDA at 30°C for 8
455 days alone (A) or in presence of strain UTB2 (B) or UTB3 (C).

456

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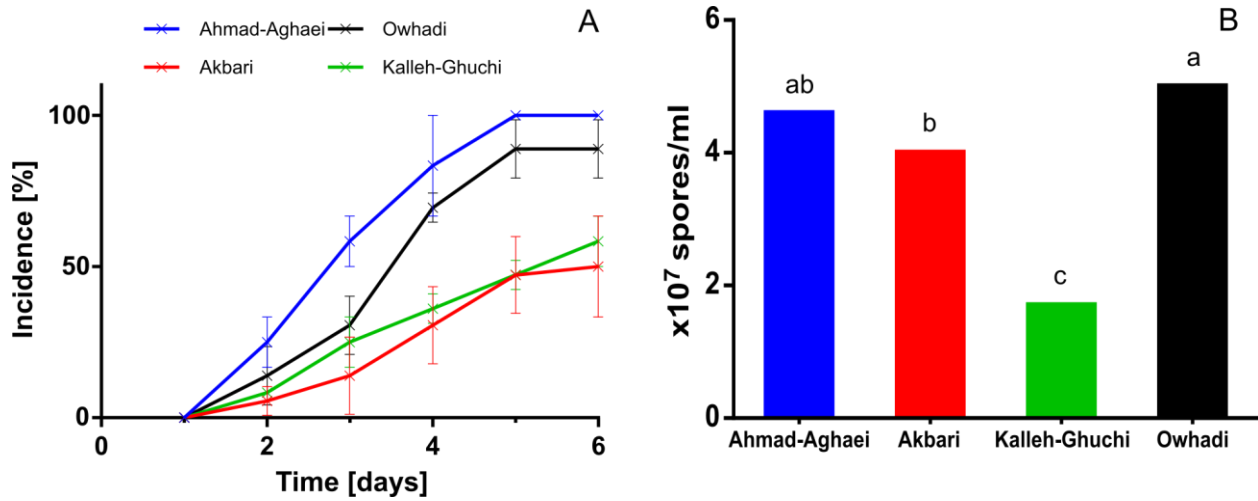


458

459 **Fig. 3** - Total aflatoxins at 6 time points (from 0 to 5 days) due to culture in PDB medium at 30
460 °C with bacterial strains UTB2 and UTB3. Initial concentration of total AFs was 200 ppb. Error
461 bars are calculated on three biological replicates.

462

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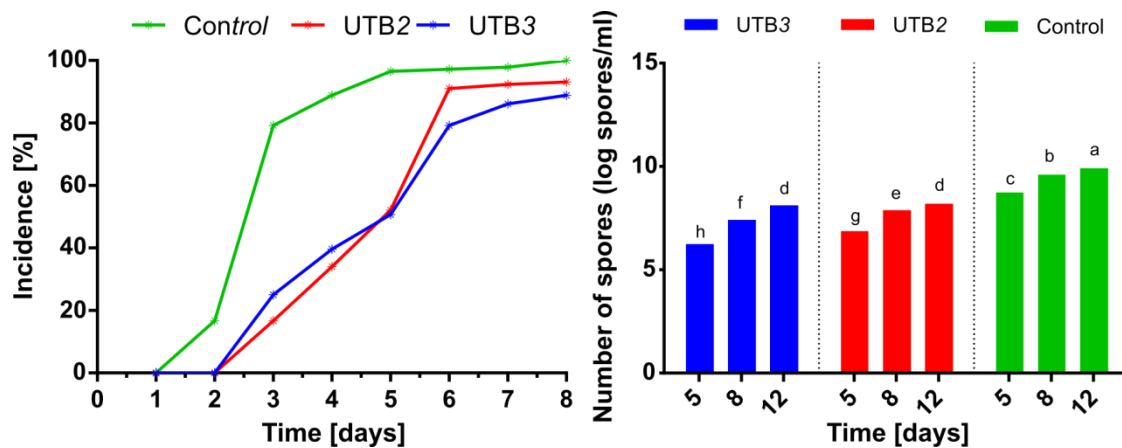
465 **Fig. 4** - Susceptibility of pistachio ‘Ahmad-Aghaei’ kernels to *A. parasiticus* mould after
466 inoculation at 30 °C and 85% relative humidity. Incidence (%) of infected wounds from 0 to 7
467 days (A). Number of spores/ml produced on the surface of pistachio kernels at 5 days of
468 inoculation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

469

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472



473

474

475 **Fig. 5** - Growth of *A. parasiticus* strain NRRL 2999 in presence of strain UTB2 (red) or UTB3
476 (blue) or alone (green) on kernels of pistachio ‘Ahmad-Aghaei’ after inoculation at 30 °C and
477 85% relative humidity. Incidence (%) of infected wounds from 0 to 8 days (A). Log (number of
478 spores/ml) produced on the surface of pistachio kernels at 5, 8 and 12 days of inoculation (B).

479