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1 **Multimycotoxin LC-MS/MS analysis in Pearl millet (*Pennisetum glaucum*) from Tunisia**

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19 **Abstract**

20 The current study assesses the natural occurrence of mycotoxins and fungal

21 metabolites in Tunisian Pearl millet. Investigations using a multi-mycotoxin liquid

22 chromatography/electrospray ionization-tandem mass spectrometric (LC/ESI-MS/MS)

23 method were carried out on a total of 220 pearl millet samples collected from farmers (n=144)

24 located in four different agroecological zones in Tunisia and from retail shops (n=76). Results

25 revealed fifty-seven regulated and emerging metabolites contaminating 91.4% of grain
26 samples. Among major mycotoxins, both aflatoxin B1 (AFB1) and ochratoxin A (OTA) were
27 the most prevalent at rate of 8.6% each and occurring at an average level of 106 and 69.4
28 µg/kg, respectively. All positives samples were significantly exceeding the European
29 thresholds (5 and 3 µg/kg, respectively). Lower incidence (5.9%) of Zearalenone (ZEA)
30 contaminated samples at levels below the authorized limits (100 µg/kg) was recorded. Pearl
31 millet grains from the south coastal zone (CT.S) had significantly the highest mean AFB1
32 contamination ($p < 0.05$). Meanwhile, OTA was encountered only in CT.S zone. Furthermore,
33 commercial samples showed higher OTA and ZEA mean concentrations than farmers samples
34 ($p < 0.05$). As regards emerging mycotoxins, *Fusarium* mycotoxins including beauvericin
35 (BEA), equisetin (EQU), monoacetoxyscirpenol (MAS) and diacetoxyscirpenol (DAS) were
36 detected in 43.2%, 43.2%, 11.4% and 10.5% of positive samples, respectively. Furthermore,
37 12.7%, 50.9%, 53.2% and 14.1% of grains were contaminated with *Alternaria* mycotoxins
38 namely alternariol (AOH), alternariolmonomethyl ether (AME), tentoxin (TEN) and
39 tenuazonic acid (TA), respectively. The majority of emerging *Fusarium* and *Alternaria*
40 mycotoxins mean levels were significantly higher in grains from north coastal area ($p < 0.01$).
41 The present study highlighted a wide range of mycotoxins in Tunisian pearl millet with a
42 different mycotoxin patterns within the agroecological zones.

43 **Keywords:** Pearl millet, LC/MS/MS-ESI, occurrence, mycotoxin, Tunisia

44 **1 Introduction**

45 Pearl millet (*Pennisetum glaucum* (L.) R. Br) is a staple multipurpose cereal crop
46 cultivated in 30 million hectares worldwide (Reddy, 2017). Actually, *P. glaucum* is an
47 annual, erect and short growing season grass, highly resistant to drought, salinity and heat
48 temperature stress. Besides, it is adapted to sandy and low fertility soils and often yields under

49 rain-fed farming (Dave et al., 1987; Haussmann et al., 2012; Pucher, Høgh-Jensen, Gondah,
50 Hash, & Haussmann, 2014). Pearl millet is able to grow and survive under harsh and adverse
51 agro-climatic conditions where more widely consumed cereals would not thrive, hence, a
52 global interest in this crop is growing concurrently with the global warming (Kodkany, et al.,
53 2013).

54 In this context, pearl millet is generally cultivated for food and fodder in arid and semi-
55 arid tropical regions constituting a major crop and one of the pillar of the agriculture sector in
56 several Asian and African countries (Jones, Liu, D Gale, Hash, & Witcombe, 1995; K. Gupta,
57 et al., 2015; Kodkany, et al., 2013; McBenedict, Percy, Kwembeya, & Maggs-Kölling, 2016).
58 Pearl millet cultivation has a lower extend in some of the non-traditional areas viz., America,
59 Australia and some European countries mainly intended for feed consumption (Martins,
60 Leticia F Pessanha, Pacheco, Avelino S. Rodrigues, & Piler Carvalho, 2018; Reddy, 2017 ;
61 Yadav & Rai, 2013).

62 Archaeobotanical investigations, highlighted that *P. glaucum* have been domesticated in
63 Sahelian Africa for 4500 years ago (Manning, Pelling, Higham, Schwenniger, & Fuller, 2011;
64 Oumar, Cedric, Pham, & Vigouroux, 2008). In north Africa, pearl millet grains have been
65 cultivated since the 8th century (Loumerem, Van Damme, Reheul, & Behaeghe, 2008), where
66 the crop is predominantly bordered in the continental Saharan area for Algeria and Libya (Ben
67 Romdhane, et al., 2018). Contrarily, in Tunisia, situated in the northern coastline of Africa,
68 pearl millet agriculture is expanded in different continental and coastal zones under diverse
69 agroclimatic conditions including humid and arid regions (Ben Romdhane, et al., 2018;
70 Ibrahima, et al., 2005; Loumerem, et al., 2008; Radhouane, 2011).

71 Nowadays, pearl millet has become increasingly attractive due to its health benefits.
72 Actually, it provides high nutritional quality characterized by high proteins, vitamins,
73 minerals and fatty acids contents in addition to its antioxidant properties. It is a gluten free

74 cereal with a low glycemic index that can be used as an alternative for coeliac and diabetic
75 diseases (Martins, Letícia F Pessanha, Pacheco, Avelino S. Rodrigues, & Piler Carvalho,
76 2018). This crop is mainly used for traditional food, mulch, forage, poultry and even for
77 making alcoholic beverages in some African countries (Appa Rao, H. Mengesha, K. Sibale, &
78 Reddy, 1986; Ben Romdhane, et al., 2018; Chilaka, Boevre, Atanda, & Saeger, 2016; Wilson,
79 et al., 2006). Mycotoxins are ubiquitous secondary metabolites produced by several fungi viz.
80 *Fusarium*, *Aspergillus*, *Penicillium*, *Claviceps*, and *Alternaria spp.*, in food and feed (Laganà,
81 2017; Šarkanj et al., 2018). The FAO/WHO Expert Committee on Food Additives, reported
82 the mycotoxins overwhelming hazards on humans and animals health by food and feed
83 consumption (JEFCA, 2001). Among the most frequently occurring mycotoxins aflatoxin B1
84 (AFB1), ochratoxin A (OTA), zearalenone (ZEA), deoxynivalenol (DON), fumonisins (FB)
85 and HT-2 have been shown to be potentially carcinogenic, genotoxic, nephrotoxic,
86 immunotoxic, teratogenic and mutagenic (Alshannaq & Yu, 2017; Marín, Cano-Sancho,
87 Sanchis, & Ramos, 2018; Smith, Madec, Coton, & Hymery, 2016; Wang, et al., 2018).
88 Recently, the emerging *Fusarium* and *Alternaria* mycotoxins have raised more awareness due
89 to their current infestation in food and feed, although in vivo toxicity and toxicokinetic data
90 are limited (Fraeyman, Croubels, Devreese & Antonissen, 2017; Gruber-Dorninger, Novak,
91 Nagl, & Berthiller 2016). In vitro studies suggest that most of emerging toxins namely
92 beauvericin (BEA), enniatins (ENNs) , alternariol (AOH), alternariolmethylether (AME),
93 moniliformin (MON), altertoxin-II (ATX-II) and stemphytoxin-III (Stemph-III) exert a
94 potent immunomodulating, mutagenic, genotoxic and cytotoxic effects on human and animal
95 cells (Fleck, et al., 2016; Fraeyman, Croubels, Devreese, & Antonissen, 2017; Gruber-
96 Dorninger, Novak, Nagl, & Berthiller, 2016; Prosperini, Juan-García, Font, & Ruiz, 2013;
97 Tedjiotsop Feudjio, et al., 2010). Further investigations associated with emerging mycotoxins
98 risk exposure revealed acute diseases like heart troubles and hemorrhages caused by

99 tenuazonic acid (TA) and MON (Fraeyman, Croubels, Devreese, & Antonissen, 2017).
100 However, no legislations have been stipulated the maximum levels for these contaminants, yet
101 (EC, 2011; 2014; 2018). Fungal growth, mycotoxin production and competitiveness of
102 mycotoxigenic species are substantially influenced by the interaction of a wide of biotic and
103 abiotic factors. Several surveys have shown the effect of geographical and environmental
104 conditions including climate, pH, light, temperature, humidity, water activity and nutrient
105 availability in the dissemination of specific toxigenic fungi and mycotoxin production under
106 propitious factors from fields to fork (Lasram, Hamdi, Chenenaoui, Mliki, & Ghorbel, 2016;
107 Magan, Hope, Cairns, & Aldred, 2003; Parra & Magan, 2004 ; Paterson & Lima, 2011 ;Van
108 der Fels-Klerx, Liu, & Battilani, 2016). The FAO has estimated that about 25% of the world's
109 crops are affected by mycotoxins each year (Devegowda, Raju, & Swang, 1998). Recent
110 investigations reported that contaminations can reach roughly 80% of cereals and feed
111 worldwide causing substantial economic losses (Kovač, Šubarić, Bulaić, Kovač, & Sarkanj,
112 2018; Streit, Naehrer, Rodrigues, & Schatzmayr, 2013). Several investigations highlighted an
113 important mycotoxins infestation in millet namely in India and Sub-Saharan African countries
114 (Chala, et al., 2014; Ezekiel, Sulyok, Warth, & Krska, 2012; R Raghavender, N Reddy, &
115 Shobharani, 2007). Up till now, in North African countries, including Tunisia, researches
116 devoted to mycotoxins contamination in pearl millet are lacking despite its importance as a
117 staple crop. Thus, the aim of this report is to assess for the first time in north Africa the
118 contamination of pearl millet intended for human consumption in Tunisia with a large
119 spectrum of mycotoxins and fungal metabolites naturally by using the trending multi-analyte
120 method by LC-MS-MS. Accordingly, to have a complete screening, samples were prospected
121 from farmers and stores in different agroecological areas in Tunisia.

122 **2 Materials and methods**

123 **2.1 Sampling**

124 A total of one hundred and forty-four of post-harvest (n=75) and post-storage (n=69)
125 pearl millet samples were randomly collected as a bulk from farmers during the campaigns
126 2014 and 2015 (Farmers samples). Generally grown up by smallholder subsistence farmers,
127 precarious environmental conditions invaded the pearl millet culture in fields and during
128 storage. Sampling was carried out in two periods. First, in September corresponding to 2-3
129 weeks after harvesting (post-harvest samples), subsequently, six months later (post-storage
130 samples). Prospecting was implemented from the main pearl millet producing regions in
131 Tunisia located in different agroecological zones (AEZs) viz., the northern, central and
132 southern regions. The coastal north zone (CT.N), including El Haouaria and Kelibia localities,
133 is characterized by a Mediterranean sub-humid climate. The coastal center (CT.C),
134 represented by Mahdia locality endowed with a higher semi-arid climate. The climate in the
135 continental center zone (CN.C), represented by Kairouan locality, is higher-arid. The coastal
136 south zone (CT.S), represented by Zarzis locality, characterized by middle arid climate. The
137 bioclimatic and geographic characteristics of the prospected sites in this study are presented in
138 (Fig.1, Table 1). Furthermore, seventy-six of pearl millet samples (n=76) were purchased
139 from local markets and grain stores (commercial samples). A total of 500 g of grains from
140 each sample was grounded in a Waring blender and stored at 4°C for multi-mycotoxin
141 analysis.

142 **2.2 Mycotoxins analysis**

143 **2.2.1 Chemicals and Reagents**

144 Methanol and acetonitrile (both LC gradient grade) were purchased from J.T. Baker
145 (Deventer, The Netherlands); ammonium acetate (MS grade) and glacial acetic acid (p.a.)

146 were obtained from Sigma–Aldrich (Vienna, Austria). Water was purified successively by
147 means of reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France).
148 Mycotoxin standard solutions were stored at –20 °C and were brought to room temperature
149 before use.

150 **2.2.2 Extraction and matrix effect**

151 Five grams of ground millet samples were extracted through 50 ml polypropylene
152 tubes (Sarstedt, Nümbrecht, Germany) with 20 ml of extraction solvent
153 (acetonitrile/water/acetic acid 79:20:1, v/v/v). After 90 min of shaking within a GFL 3017
154 rotary shaker (GFL, Burgwedel, Germany), samples were transferred to vials and diluted with
155 an equal volume of the dilution solvent (acetonitrile/water/acetic acid 20:79:1, v/v/v). 5 µl of
156 the diluted extracts were subsequently injected into an LC-MS/MS system (Sulyok, Berthiller,
157 Krska, & Schuhmacher, 2006). For spiking experiment, 0.25g of four blanks millet samples,
158 were inoculated with mastermix standards and used for extraction. To allow evaporation of
159 the solvent and to establish equilibration between the analytes and the matrix, the spiked
160 samples were mixed and stored at room temperature overnight in the dark.

161 **2.2.3 LC/ MS-MS parameters**

162 Analysis were carried out via a developed multimycotoxin LC/MS-MS method
163 (Malachová, Sulyok, Beltrán, Berthiller, & Krska, 2014) that has been updated and extended
164 to cover 650 analytes. The multi-analyte LC/MS/MS method based on a “dilute and shoot”
165 approach was used for the determination of various fungal and bacterial metabolites which is
166 optimized and validated according to the guidelines established in the Directorate General for
167 Health and Consumer Affairs of the European Commission SANCO document No.
168 12495/2011 (SANCO, 2011). Briefly, analyses were performed with a Qtrap 5500 LCMS/MS
169 System (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ion Spray
170 electrospray ionization (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn,

171 Germany). Chromatographic separation was undertaken at 25°C on a Gemini C18-column,
172 150X 4.6 mm i.d., 5 µm particle size, equipped with a C18 4 X 3 mm i.d. security guard
173 cartridge (Phenomenex, Torrance, CA, USA). ESI-MS/MS was performed in the time-
174 scheduled multiple reaction monitoring (MRM) mode, both in positive and negative
175 polarities, in two separate chromatographic runs per sample, by scanning two fragmentation
176 reactions per analyte. The MRM detection window of each analyte was set to its expected
177 retention time ± 27 s and ± 48 s in the positive and negative mode, respectively. Confirmation of
178 positive analyte identification was obtained by the acquisition of two MRMs per analyte (with
179 the exception of moniliformin and 3-nitropropionic acid that exhibit only one fragment ion).
180 This yielded 4.0 identification points according to European Union Commission decision
181 2002/657 (EC, 2002). In addition, the LC retention time and the intensity ratio of the two
182 MRM transitions agreed with the related values of an authentic standard within 0.1 min and
183 30% relative, respectively. Quantification was performed using an external calibration based
184 on serial dilutions of a multi-analyte stock solution. Results were corrected for apparent
185 recoveries determined by spiking experiments. The expanded measurement uncertainty has
186 been determined to be 50% (Stadler, Sulyok, Schuhmacher, Berthiller & Krska, 2019). The
187 accuracy of the method is verified on a routine basis by participation in interlaboratory testing
188 schemes including a broad variation of matrices of grains, nuts, dried fruits, spices, baby food
189 and animal feed. Satisfactory z-scores between -2 and 2 have been obtained for more than
190 94% of higher than 1000 results submitted so far.

191 **2.3 Statistical analysis**

192 Statistical analyses were performed with SAS software version 9.2. In order to study the
193 significance of mycotoxins contamination in pearl millet across different agroecological
194 zones, sampling provenance, years, and sampling periods, data were analyzed using non-
195 parametric tests Mann Whitney and Kruskal Wallis test followed by a Post Hoc Dunn test.

196 Analysis were realized taking into consideration positive and negative samples. P-values<0.05
197 were considered statistically significant.

198 **3 Results**

199 **3.1 Major mycotoxins**

200

201 A total of 57 metabolites were quantified in pearl millet samples (Table 2). Out of the
202 total number of different metabolites detected, major mycotoxins and derivatives, *Fusarium*
203 metabolites, Aflatoxin precursors, *Alternaria* metabolites were accounted for 15.8%, 19.2%,
204 8.7% and 14%, respectively. Other *Aspergillus* and *Penicillium* metabolites, were detected
205 with an incidence of 8.7% and 12.3% of the total metabolites, respectively. Some bacterial
206 metabolites and metabolites from unspecified sources were also observed at frequency of 5.2
207 to 15.8% out of the total number of different metabolites detected, respectively. Otherwise,
208 about 91.4% (201/220) of pearl millet samples were contaminated by at least one analyte and
209 up to 23 metabolites per sample. Statistical analysis, revealed that the sampling years (2014
210 and 2015) as well as the sampling period (post-harvest and post-storage) had no significant
211 effect on pearl millet contamination with major mycotoxins namely AFs, OTA and ZEA
212 ($p>0.05$). Meanwhile, significant differences in pearl millet contamination have been
213 observed across agroecological zones and samples provenance (farmers and commercial) for
214 several mycotoxins including AFB1, ZEA, AOH, TEN, BEA, DAS, MAS and EQUUS
215 ($p<0.05$).

216 Among major mycotoxins, aflatoxins, namely aflatoxin B1 (AFB1) were the most
217 prevalent. Out of 10% of pearl millet samples contaminated with aflatoxins (22/220), 86.4%
218 (19/22) contained AFB1 at an average of 106 $\mu\text{g}/\text{kg}$ and a median of 29.6 $\mu\text{g}/\text{kg}$ in positive
219 millet samples. Taking into consideration all samples, the mean contamination of millet with
220 AFB1 was about 9.2 $\mu\text{g}/\text{kg}$. Table 3 presents the mycotoxins levels in pearl millet according

221 to agroecological zones. Statistical analysis showed significant differences ($p < 0.05$) for AFB1
222 contamination in pearl millet across AEZs between CT.N and CT.C comparing to CT.S and
223 CN.C zones, respectively. Results revealed clearly highest levels in the CT.S zone (57.8
224 $\mu\text{g}/\text{kg}$ mean and 1046 $\mu\text{g}/\text{kg}$ maximum) comparing to the CT.N (0.53 $\mu\text{g}/\text{kg}$ mean and 36.6
225 $\mu\text{g}/\text{kg}$ maximum) and CN.C zones (9.67 $\mu\text{g}/\text{kg}$ mean and 245 $\mu\text{g}/\text{kg}$ maximum). However,
226 none of the samples deriving from the coastal center contained aflatoxins at detectable levels.
227 Comparing the sample provenance, no statistical significant difference ($p > 0.05$) was found in
228 AFB1 contamination between farmers and commercial samples with mean level of 11.4 and
229 5.2 $\mu\text{g}/\text{kg}$, respectively. However, the incidence of AFB1 was higher in commercial (13.2%)
230 than in farmers' millet samples (6.3%) with mean concentration of positive samples about
231 39.4 $\mu\text{g}/\text{kg}$ (117 $\mu\text{g}/\text{kg}$ maximum) and 181 $\mu\text{g}/\text{kg}$ (1046 $\mu\text{g}/\text{kg}$ maximum), respectively.
232 Aflatoxin B2 (AFB2) and aflatoxin M1 (AFM1), were, respectively, present in only one
233 farmers' samples (0.7%) with levels of 96.1 and 18.1 $\mu\text{g}/\text{kg}$. Aflatoxin G1 (AFG1) was also
234 detected in only one commercial sample with an amount of 20.3 $\mu\text{g}/\text{kg}$.

235 Ochratoxin A (OTA) was detected in 8.6% (19/220) out of positive pearl millet samples
236 with mean level of positive samples of 69.4 $\mu\text{g}/\text{kg}$ and 36 $\mu\text{g}/\text{kg}$ median. With respect to
237 negative samples, the average level was 6 $\mu\text{g}/\text{kg}$. Statistical analysis showed no significant
238 differences in OTA contamination across agroecological zones ($p > 0.05$). Nonetheless, this
239 mycotoxin occurred only in center continental and south coastal zones while in coastal north
240 and center areas no OTA was detected. Otherwise, OTA showed significantly highest
241 amounts ($p < 0.0001$) in commercial samples compared to farmers' samples with mean level of
242 12 and 2.83 $\mu\text{g}/\text{kg}$, respectively. In fact, OTA was observed in 19.7% of commercial samples
243 with mean amount of 60.6 $\mu\text{g}/\text{kg}$ (231 $\mu\text{g}/\text{kg}$ maximum) for positives samples, while, in
244 farmers' samples, OTA was detected at low frequency (2.8%) with high levels (102 $\mu\text{g}/\text{kg}$
245 mean, 178 $\mu\text{g}/\text{kg}$ maximum of positives samples).

246 Zearalenone (ZEA) was present in 5.9% (13/220) out of total grain samples with mean
247 and median ZEA levels of positive samples of 16 and 12.2 µg/kg, respectively. Statistical
248 analysis revealed significant higher levels in commercial samples than in farmers' samples
249 ($p < 0.001$). In fact, commercial samples were the most contaminated by ZEA with a frequency
250 of 15.8% and occurred at 16.3 µg/kg on average (40.2 µg/kg maximum). However, only one
251 sample of farmers' samples was contaminated with ZEA at lower concentrations (13.1 µg/kg).
252 It's worthy to emphasize that, the levels of ZEA contamination in millet samples detected in
253 our investigations were under the limits set up by the European committee (100 µg/kg for raw
254 cereals). Nivalenol (NIV) was observed only in commercial samples at low incidence (2 out
255 of 220 samples) with positive samples mean concentration of 114 µg/kg and maximum of 161
256 µg/kg.

257 **3.2 Emerging metabolites**

258 Our findings revealed, in addition to major mycotoxins, the presence of several
259 metabolites including masked and emerging mycotoxins, usually known as conjugated and
260 unregulated mycotoxins, respectively, viz., *Alternaria*, *Fusarium* and *Aspergillus* mycotoxins
261 (Kovač, et al., 2018) (Table 2).

262 *Fusarium* mycotoxins were encountered in the current study, monoacetoxyscirpenol
263 (MAS) and diacetoxyscirpenol (DAS), occurred in 11.4 and 10.5% of samples at mean levels
264 of 126 µg/kg (339 µg/kg maximum) and 61.5 µg/kg (168 µg/kg maximum) in positive
265 samples, respectively. Overall, a statistical significant difference in means of both analytes
266 across AEZs was observed ($p < 0.01$) with greater contamination in the CT.N zone with
267 average levels of 43.4 and 20.1 µg/kg for MAS and DAS, respectively. As regards sampling
268 provenance, higher means concentrations (20.8 and 9.64 µg/kg, respectively) were detected in
269 farmers' samples ($p < 0.01$). Beauvericin (BEA) and equisetin (EQU) were the most prevalent
270 at frequency of 43.2% each. Beauvericin was generally detected at trace levels with 0.87

271 $\mu\text{g}/\text{kg}$ on average and reached maximum of 13 $\mu\text{g}/\text{kg}$ in positive millet samples. The
272 beauvericin contamination was significantly different within AEZs ($p < 0.01$) with highest
273 amounts in the CT.N zone comparing to other areas. As concern commercial samples, BEA
274 was present at the same levels than farmers samples ($p > 0.05$) with 1 $\mu\text{g}/\text{kg}$ mean (5 $\mu\text{g}/\text{kg}$
275 maximum). Equisetin occurred at higher amounts with mean concentration of 142 $\mu\text{g}/\text{kg}$ and
276 up to 1909 $\mu\text{g}/\text{kg}$ out of the positive millet samples. Statistical data showed a significant
277 difference across AEZs, sampling provenance as well as sampling years ($p < 0.01$). Farmer
278 samples sourced from the CT.N zone were the most contaminated (149 $\mu\text{g}/\text{kg}$ mean and 1909
279 $\mu\text{g}/\text{kg}$ maximum). In addition, out of the total farmers' samples, grains collected in 2015 had
280 significantly greater equisetin and beauvericin amounts than in 2014, with mean values of 124
281 and 0.52 $\mu\text{g}/\text{kg}$, respectively ($p < 0.01$). Otherwise, equisetin levels in commercial millet
282 samples (34 $\mu\text{g}/\text{kg}$ mean and 588 $\mu\text{g}/\text{kg}$ maximum) were significantly inferior to farmers'
283 grains (75.9 $\mu\text{g}/\text{kg}$ mean). Enniatins (ENNs) were also present in 2.72% of the samples with
284 an incidence of 1.8% for enniatin B at trace level with 1.58 $\mu\text{g}/\text{kg}$ mean of positive samples.
285 Enniatin A1 and B1 were observed in only one sample, whereas, enniatin A was not detected.
286 No significant differences in the mean concentrations found in samples collected across the
287 four AEZs and sampling provenance ($p > 0.05$). Fusapyron and deoxyfusapyron, were also
288 present at an average level of 113 $\mu\text{g}/\text{kg}$ (376 $\mu\text{g}/\text{kg}$ maximum) and 34.3 $\mu\text{g}/\text{kg}$ (83.5 $\mu\text{g}/\text{kg}$
289 maximum), respectively, in positive samples. Data analysis showed a significant difference
290 for fusapyron and deoxyfusapyron contamination within farmers and commercial samples
291 ($p < 0.0001$). Higher amounts were recovered in millet collected from grain stores with an
292 average concentration of 31.6 $\mu\text{g}/\text{kg}$ (376 $\mu\text{g}/\text{kg}$ maximum) and 23.7 $\mu\text{g}/\text{kg}$ (83.5 $\mu\text{g}/\text{kg}$
293 maximum), for fusapyron and deoxyfusapyron, respectively.

294 *Alternaria* metabolites occurred with an incidence reaching 13.8% out of the total
295 analytes detected. Contamination frequencies with alternariol (AOH), alternariolmethylether

296 (AME), tentoxin (TEN), tenuazonic acid (TA) and macrosporin (MAC) were 12.7, 50.9, 53.2,
297 14.1 and 47.3% of the total millet samples, respectively. AOH average contamination within
298 positive samples was 52.1 µg/kg and a median value of 26.9 µg/kg. With reference to
299 negative samples, mean level was about 6.6 µg/kg. Statistical analysis highlighted a
300 significant difference ($p < 0.01$) in AOH levels across AEZs with higher contamination in
301 CT.N zone (12.9 µg/kg mean and 194 µg/kg maximum) comparing to CT.C, CT.S and CN.C
302 zones (Table). According to sampling provenance, no significant difference in AOH amounts
303 was identified with mean levels of 7 µg/kg (194 µg/kg maximum) and 5.9 µg/kg (130 µg/kg
304 maximum) in farmers' and commercial samples, respectively. Similarly, to AOH, AME
305 contamination was significantly higher ($p < 0.001$) in CT.N area (66.8 µg/kg mean and 777
306 µg/kg maximum) comparing to the other regions. Within 112 contaminated samples, the mean
307 AME level encountered was 94.2 µg/kg (1351 µg/kg maximum). Besides, commercial
308 samples (71.5 µg/kg mean) were significantly ($p < 0.05$) more contaminated than farmers
309 samples (35.5 µg/kg mean). Regarding TEN and TA analytes, the average values of positive
310 samples were 14.5 and 98.1 µg/kg, respectively. Taking into account the negative samples,
311 statistical difference was identified only for TEN within farmers and commercial samples
312 ($p < 0.0001$) with the mean levels of 9.3 and 4.6 µg/kg, respectively. In concordance with
313 *Alternaria* metabolites, AOH and AME, statistical analysis of TEN amounts in farmer's
314 samples showed that millet grains originated from CT.N zone were the most contaminated
315 with mean concentration of 16.6 µg/kg and up to 111 µg/kg ($p < 0.01$). Conversely, no
316 significant differences were observed across the four AEZs for TA contamination. Statistical
317 analysis of data relative to *Alternaria* emerging mycotoxins, revealed that sampling year as
318 well as sampling period had a significant difference in pearl millet contamination, only for
319 TEN and TA ($p < 0.05$). In fact, pearl millet prospected in 2014 campaign, contained higher
320 amounts of TEN and TA with mean values of 15.1 µg/kg (111 µg/kg maximum) and 39.5

321 $\mu\text{g}/\text{kg}$ (505 $\mu\text{g}/\text{kg}$ maximum), respectively. Whereas, in 2015, lower levels were found in
322 millet seeds for TEN (4.42 $\mu\text{g}/\text{kg}$ mean), while no TA has been detected within all samples.
323 Additionally, post-storage samples were significantly more infested with TEN (12.6 $\mu\text{g}/\text{kg}$
324 mean and 111 $\mu\text{g}/\text{kg}$ maximum). Whereas, TA levels were statistically higher in post-harvest
325 pearl millet (28.6 $\mu\text{g}/\text{kg}$ mean and 505 $\mu\text{g}/\text{kg}$ maximum). Macrosporin was also detected in
326 millet seeds showing a statistical difference in means across AEZs ($p < 0.01$) viz., the CN.C vs
327 CT.S and the CT.C vs the CT.N zone. Highest amounts were highlighted in the CT.N zone
328 reaching 2302 $\mu\text{g}/\text{kg}$. In commercial samples, lower levels of MAC were detected with 31.2
329 $\mu\text{g}/\text{kg}$ mean and 438 $\mu\text{g}/\text{kg}$ maximum. Altersetin and infectopyron were also found only in
330 farmers' samples with mean concentrations of 32.9 and 318 $\mu\text{g}/\text{kg}$, respectively.

331 Apart from these analytes, there was a broader pattern of *Aspergillus* metabolites
332 reported in pearl millet including 3-nitropropionic acid, malformin A and C, agroclavine, and
333 the kojic acid (KA). This latter, occurred at frequency of 7.7% of grains with an average in
334 positive samples of 7992 $\mu\text{g}/\text{kg}$ (41649 $\mu\text{g}/\text{kg}$ maximum) while, taking into consideration the
335 negative samples mean amount was 617 $\mu\text{g}/\text{kg}$. A statistically significant difference in mean
336 KA contamination was identified within the four AEZs ($p < 0.01$) with the highest infestation
337 in the CN.C zone (2651 $\mu\text{g}/\text{kg}$ mean). Besides, five Aflatoxin precursors were also recovered
338 such as sterigmatocystin, averantin, averufin and versicolorins (VER A and VER C). Some
339 *Penicillium* metabolites were observed like curvularin and dihydrocitrinone at levels reaching
340 200 and 2851 $\mu\text{g}/\text{kg}$, respectively. Additionally, citrinin (CIT) was only encountered in
341 commercial samples (6.6%) with an average amount of 6921 $\mu\text{g}/\text{kg}$ and up to 25663 $\mu\text{g}/\text{kg}$.
342 Among the nine metabolites from unspecified sources detected in pearl millet, monocerin was
343 the most prevalent (47.3%) with an average concentration of 259 $\mu\text{g}/\text{kg}$ and 2142 $\mu\text{g}/\text{kg}$
344 maximum. Statistical differences were observed across sampling provenance ($p < 0.05$) with
345 higher amounts in commercial samples (218 $\mu\text{g}/\text{kg}$ mean). Asperglaucide were also found in

346 37.3% of millet occurring at levels of 77.6 µg/kg mean and 1275 µg/kg maximum.
347 Xanthotoxin, a plant toxin, was observed in 4.5% of the total pearl millet samples.
348 Xanthotoxin contamination was significantly different across AEZs ($p < 0.05$) with highest
349 amount in the CT.N zone (20.8 µg/kg mean and 802 µg/kg maximum). Furthermore, cyclo
350 (L-Pro-L-Tyr), neoechinulin A, emodin, asperphenamate, usnic acid and lotaustralin were
351 also detected. Bacterial metabolites like chloramphenicol, ascochlorin and antibiotic PF 1052
352 were also found in the current study. Meanwhile, nigragilin was also recovered in millet, but
353 due to a lack of standards, the quantification was not possible.

354 **4 Discussion**

355 The present study provides the assessment of a large pattern of mycotoxins and several
356 derivatives in pearl millet and even of less often investigated fungal and bacterial metabolites
357 for the first time in a North African country. The detection of this border range of secondary
358 metabolites is due to the high sensitivity and accuracy of the LC/MS-MS for multi-
359 mycotoxins analysis. This approach based on the analysis of diluted crude extracts without
360 any previous sample pretreatments, can cover till 620 analytes (Sulyok, Krska, &
361 Schuhmacher, 2007; 2010). By far not all of the detected compounds are toxicologically
362 relevant at their naturally occurring levels and seems to be of little or no health concern to
363 consumers. The present study might disclose the remaining knowledge and research gaps for
364 these compounds naturally occurring in pearl millet in order to have an overview of grains
365 contaminants which might support an efficient assessment. Thus, this work affords to have a
366 spectrum of mycotoxins in pearl millet and assesses the threat of humans and animals
367 exposure to these metabolites through the food and feed consumption.

368 The multiple mycotoxins occurrence recently echoed and evoked interest in several
369 surveys carried out in different commodities, namely cereals and cereals products. Our
370 finding emphasized the multi-toxin co-occurrence in Tunisian pearl millet. Hence, that might

371 lead to more severe effects on humans and animals health and can have antagonist, additive or
372 synergic effect (Abia, et al., 2013; Adetunji, Atanda, Ezekiel, & Ogara, 2014; Chilaka,
373 Boevre, Atanda, & Saeger, 2016; Ezekiel, et al., 2014; Smith, Madec, Coton, & Hymery,
374 2016; Wang, et al., 2018). Mycotoxins distribution specially with respect to different
375 agroecological zones in Tunisia as well as the sampling provenance was clearly observed in
376 the current study. Overall, according to the obtained results, Tunisian pearl millet were highly
377 contaminated by a myriad of secondary metabolites of which *Alternaria* and *Fusarium*
378 metabolites were the most prevalent. Whereas, *Aspergillus* and *Penicillium* metabolites
379 occurred at lower incidence.

380 Among the regulated mycotoxins detected in the present study, aflatoxins levels
381 detected in all pearl millet positive samples were significantly above the thresholds
382 established by the European commission (EC, 2006) for cereals and its derivatives (2 µg/kg
383 for AFB1 and 5 µg/kg for the sum of AFB1, B2, G1 and G2), namely the AFB1 which is
384 considered as the most toxic in the aflatoxins group. In our findings, albeit the medium
385 frequency of AFB1 in samples (8.6%), high amounts were recovered in pearl millet ranging
386 from 12.2 to 1046 µg/kg. It is worth mentioning, the harmful hazards that may affect human
387 safety. Higher prevalence of AFB1 was observed in Kenyan millet (64%) with a wide range
388 levels from <1 to 1658 µg/kg. However, only 10% of samples were above the standards limits
389 (Sirma, 2016). Our data showed clearly higher levels of AFB1 comparing to comparative
390 surveys in United State of America and Guinea on different cereals that highlighted the
391 presence of AFB1 in pearl millet at lower concentrations than maize and sorghum. A maximal
392 concentration of 7.1 µg/kg was detected in pearl millet grown in dryland conditions at Tifton,
393 Georgia, USA (Wilson et al., 2006). While, the average of AFB1 contamination in Guinean
394 pearl millet was about 4.6 µg/kg (Bandyopadhyay, Kumar, & Leslie, 2007). A Nigerian
395 survey on the fonio millet revealed very low levels of AFB1 up to 1.4 µg/kg, in spite of their

396 high incidence of 81% (Ezekiel, Sulyok, Warth, & Krska, 2012). This in agreement with
397 results reported by Chala et al., (2014) showing that Ethiopian finger millet is less prone to
398 aflatoxins contamination comparing to sorghum (1.12 and 29.5 $\mu\text{g AFB1/kg}$ on average,
399 respectively). This was previously approved by (Alpert, Hutt, Wogan, & Davidson, 1971) in
400 tropical areas where contaminated millet were lower (up to 100 $\mu\text{g/kg}$) than in sorghum (up to
401 1000 $\mu\text{g/kg}$). In the current study, AFB2, AFG1 and AFM1 were detected once at high levels
402 (91.6, 20.3 and 18.1 $\mu\text{g/kg}$, respectively). The AFM1 and AFB2 co-occurred in the sample
403 containing the highest level of AFB1 (1046 $\mu\text{g/kg}$). The presence of AFM1 in pearl millet
404 grains might be due to high levels of AFB1 (Chala, et al., 2014; Yabe, et al., 2012). In
405 compliance with our results Penugonda, Sivadevuni, and M Reddy (2010) reported that AFG1
406 and AFM1 have been detected in addition to AFB1 in Indian finger millet. However, only
407 AFB1 and AFG1 were observed in finger millet from Ethiopia in addition to trace levels of
408 AFM1 ($<1 \mu\text{g/kg}$) (Chala, et al., 2014).

409 To date, OTA was not encountered in other previous investigations about millet
410 (Bandyopadhyay, Kumar, & Leslie, 2007 ; Chala, et al., 2014 ; Ezekiel, Sulyok, Warth, &
411 Krska, 2012). In concordance with AFB1, our results showed medium incidence (8.6%) of
412 OTA with levels ranging between 16.2 and 231 $\mu\text{g/kg}$. Besides, in compliance with
413 aflatoxins, levels of OTA detected in millet grains were also beyond the legal limits permitted
414 by the European Commission for raw cereals (5 $\mu\text{g/kg}$). In line with millet, sorghum have
415 been alluded to be an African indigenous staple crop intended for human consumption.
416 Conversely to millet, sorghum have been reported to be more prone to OTA contamination. In
417 fact, Ghali et al., (2008), found that 52% of the Tunisian sorghum samples were contaminated
418 with mean OTA concentration of 14.4 $\mu\text{g/kg}$ (up to 36.4 $\mu\text{g/kg}$). Ayalew, Fehrmann, Lepschy,
419 Beck, and Abate (2006) reported high OTA levels in sorghum (up to 2106 $\mu\text{g/kg}$) from
420 Ethiopia comparing to Barely, Wheat and Teff.

421 In our survey, 5.9% of zearalenone contaminated grains showed concentrations below the
422 regulation limits (100 µg/kg in raw cereals) with levels ranging from 4.16 to 40.2 µg/kg.
423 Investigations in Nigeria on mouldy sorghum showed a lesser extent of OTA contamination
424 comparing to AFB1 and Zearalenone that reached 255 µg/kg (Makun, Gbodi Timothy,
425 Akanya, Salako Ezekiel, & Ogbadu Godwin, 2009). However, among the wheat, barely, teff
426 and sorghum analyzed in Ethiopia, only 6.9% of ZEA were encountered in harvest sorghum
427 grain with maximum levels of 32 µg/kg (Ayalew, Fehrmann, Lepschy, Beck, & Abate, 2006).
428 Similar reports in Nigerian fonio millet showed low frequency of Zearalenone contamination
429 with one sample exceeding the European limits (987 µg/kg).

430 It's important to note that no more other major mycotoxin viz. Deoxynivalenol (DON),
431 Fuminosins (FB) and HT-2 has been detected in our study, despite their prevalence and
432 toxicity in other investigations on millet (Chala, et al., 2014; Chilaka, Boevre, Atanda, &
433 Saeger, 2016; Ezekiel, Sulyok, Warth, & Krska, 2012). In Nigeria, Chilaka, Boevre, Atanda,
434 and Saeger (2016) alluded the occurrence of Fumonisin B1, B2 and B3 (Σ FB) in addition to
435 HT-2 and DON in millet and sorghum with generally high concentrations. FB amounts were
436 around 83 and 2113 µg/kg while DON levels were about 100 and 151 µg/kg in sorghum and
437 millet, respectively. Lower frequencies and concentrations were observed in HT-2
438 contaminated samples for both crops (20 and 36 µg/kg mean in sorghum and millet,
439 respectively). This result could be related to a probable low presence of *Fusarium* species
440 producing those mycotoxins in Tunisian millet.

441 A part from unregulated mycotoxins, defined as emerging mycotoxins, *Fusarium* and
442 *Alternaria* emerging mycotoxins were the most prevalent in our findings. Even though,
443 seldom toxicological data are recognized about these emerging mycotoxins, to date, but we
444 could not ignore the potential threat they can present to the consumer health (Abia, et al.,

445 2013; Berthiller, et al., 2013; Fraeyman, Croubels, Devreese, & Antonissen, 2017; Gruber-
446 Dorninger, Novak, Nagl, & Berthiller, 2016; Kovač, et al., 2018; Oyedele, et al., 2017).

447 Among the Fusariotoxins, DAS and MAS, a type A trichothecenes, are considered to be
448 one of the most toxic trichothecenes mainly causing emesis and haematotoxicity effects (EC,
449 2018). In our survey, DAS and MAS occurred at higher frequencies (10.5 and 11.4%) and
450 amounts (61.5 and 126 µg/kg mean), respectively, comparing with those found in only one
451 sample of Nigerian finger millet (1.43 and 2.72 µg/kg mean, respectively) (Chala, et al.,
452 2014). However, Chilaka, Boevre, Atanda, and Saeger (2016) detected greater frequency of
453 DAS in millet (29%) but at lower level (5 µg/kg mean). Same concentration was observed in
454 sorghum but at frequency of 18%. Similar analysis on Nigerian sorghum showed higher
455 prevalence (40 and 35.7%) even though at levels lower than those occurring in our findings
456 (11.9 and 16.3 µg/kg mean) for DAS and MAS, respectively (Chala, et al., 2014). Unless the
457 lack of regulatory limits for DAS, it has been reported to be extremely toxic for human and
458 animals health. Although, it's worth to note that an acute reference dose (ARfD) of 3.2 µg
459 DAS/kg body weight (bw) and a tolerable daily intake (TDI) of 0.65 g DAS/kg bw were
460 established (Chilaka, Boevre, Atanda, & Saeger, 2016; EC, 2018). Enniatins especially known
461 for their cytotoxic and apoptotic effects in human cells (Gruber-Dorninger, Novak, Nagl, &
462 Berthiller, 2016; Sulyok, Krska, & Schuhmacher, 2007; Tedjiotsop Feudjio, et al., 2010),
463 were recovered in our surveys at trace levels reaching maximum for enniatin B (1.8%; 4.38
464 µg/kg). Moreover, only 0.5% of positive samples were contaminated with Enniatin B1 and A1
465 with amounts as low as a few µg/kg (0.71 and 0.78 µg/kg, respectively). However, no
466 Enniatin A was detected. Conversely, Chala et al., (2014) found higher incidence of ENNs in
467 Nigerian finger millet (47.1, 44.3, 38.6 and 21.4% for ENB, B1, A1 and A, respectively), but
468 similarly with low concentrations (<1 µg/kg). According to Ezekiel, Sulyok, Warth, and
469 Krska (2012) no enniatins were recorded in Nigerien fonio millet. However, enniatins has

470 been reported to contaminate other cereals from North Africa at higher levels of up to 480
471 mg/kg in sorghum (Oueslati, Meca, Mliki, Ghorbel, & Mañes, 2011) and 688 µg/kg in cereal
472 products (Fraeyman, Croubels, Devreese, & Antonissen, 2017). Thus, the pearl millet in
473 Tunisia is less prone to enniatins contamination. The low concentrations detected in this study
474 might not have any consequences for human health. However, to date, we cannot assert the
475 effect of their chronic exposure on human health due to the lack of relevant toxicity data in
476 vivo (Gruber-Dorninger, Novak, Nagl, & Berthiller, 2016; Sulyok, Krska, & Schuhmacher,
477 2010).

478 In the current study, beauvericin well in line with equisetin highly occurred in pearl
479 millet seeds at incidence of 43.2% for both analytes. Nonetheless, lowest amounts were
480 observed for BEA (0.09 -13 µg/kg range). Similar concentrations (9.15 µg/kg mean) were
481 detected in 100% of finger millet samples from Nigeria, while 71.4% of sorghum samples
482 were BEA contaminated (17 µg/kg mean) (Chala, et al., 2014). In Tunisia, authors reported
483 no BEA contamination in sorghum (Oueslati, Meca, Mliki, Ghorbel, & Mañes, 2011). EQU
484 concentrations in millet found in this work (142 µg/kg on average) were generally higher than
485 Nigerian finger millet and sorghum with mean concentrations of 49 and 60.5 µg/kg, in 72.7
486 and 31.4% of samples, respectively (Chala, et al., 2014). Ezekiel, Sulyok, Warth, and Krska
487 (2012) reported greater EQU levels with 320 µg/kg on mean in fonio millet.

488 This report also revealed Fusapyron and Deoxyfusapyron occurrence in pearl millet,
489 defined as antifungal metabolites, they were essentially found in commercial samples whereas
490 they were encountered in previous surveys on different cereals and commodities (Getachew,
491 et al., 2018; Oliveira, Rocha, Sulyok, Krska, & A. Mallmann, 2016).

492 As regards the emerging *Alternaria* mycotoxins, tentoxin was the most common in pearl
493 millet, contaminating roughly half of sampled millet in the range of 2.1 to 111 µg/kg. While,
494 in Nigerian finger millet TEN was present in all samples but at lower concentration up to 15.5

495 $\mu\text{g}/\text{kg}$ (Chala, et al., 2014). Other investigations revealed 77% of TEN infested cereals from
496 China ranged from 0.4 to 258 $\mu\text{g}/\text{kg}$ (Fraeyman, Croubels, Devreese, & Antonissen, 2017).
497 AME and MAC were also omnipresent in our findings (50.9 and 47.3%) with maximum
498 concentrations of 1351 and 2302 $\mu\text{g}/\text{kg}$, respectively. According to Chala et al., (2014),
499 concentration levels observed in finger millet were far lower, around 10 and 100-fold less, for
500 AME and MAC, respectively.

501 AOH and TA were notably detected in our investigations (12.7 and 14.1%, respectively)
502 showing high levels with maximum concentration of 194 and 505 $\mu\text{g}/\text{kg}$, respectively.
503 Whereas, Ezekiel, Sulyok, Warth, and Krska (2012) alluded lesser contaminated Nigerian
504 fonio millet samples with severely lower amounts of AOH (0.3 - 10 $\mu\text{g}/\text{kg}$ range) and greater
505 levels for TA infestation (14 - 1049 $\mu\text{g}/\text{kg}$ range). Furthermore, maximum levels of up 832
506 and 4224 $\mu\text{g}/\text{kg}$ for AOH and TA, respectively, have been reported in raw cereals from
507 Europe (Fraeyman, Croubels, Devreese, & Antonissen, 2017).

508 Hence, the variety and co-occurrence of *Alternaria* and *Fusarium* emerging mycotoxins
509 in Tunisian pearl millet might postulate the presence of several mycotoxigenic fungal species.
510 This pose threat to consumer health due to their cytotoxic and mutagenic effects leading to
511 chronic diseases, although no legislations have been established yet.

512 Regarding toxins contamination across the sampling provenance, our outcomes
513 demonstrated different contamination levels and frequencies for some metabolites comparing
514 farmers and commercial samples. Among major mycotoxins, OTA was significantly more
515 detected in commercial samples ($p < 0.05$). In fact, wrong post-harvest conditions
516 (temperature, humidity, water activity, aeration) lead to peaks of ochratoxigenic moulds
517 mainly, *Aspergillus* and *Penicillium* species. Nevertheless, it's important to emphasize that
518 high OTA amounts were recorded in few farmers samples. Contrarily, AFB1 was detected at
519 high levels in farmers as well as in commercial samples with no significant difference.

520 Noteworthy, OTA and AFB1, generally classified as storage mycotoxins, were present in
521 freshly harvested samples (2-3 weeks after harvesting) with levels up to the authorized limits.
522 Hence, this is probably due to an inadequate drying of millet grains before storage which
523 enhance mycotoxigenic fungi proliferation and their toxinogenic potential. Otherwise, it's
524 worth to note that in the present finding, contamination of pearl millet with major mycotoxins
525 was similarly observed in post-harvest as well as in post storage samples. Notwithstanding,
526 several unregulated mycotoxins including *Fusarium* (EQUUS, DAS and MAS) and *Alternaria*
527 mycotoxins (TEN, AME) were significantly greater in farmers pearl millet samples.
528 Nonetheless, no conclusion involving the effect of storage on mycotoxins contamination in
529 pearl millet could be drawn since the analyzed samples are originated from different sources.

530 To further get insights on toxin variation across different AEZs, levels of some regulated
531 mycotoxins in millet showed geographical variations. Our finding, clearly revealed that AFB1
532 mean levels in millet from the coastal south and continental center area were significantly
533 higher than those from the coastal north and center ($p < 0.05$). Furthermore, OTA was detected
534 only in millet samples originated from coastal south and continental center. Whilst, the kojic
535 acid, an *Aspergillus* metabolite, was significantly detected in millet from the continental
536 center area. In contrast, the overhanding majority of *Fusarium* and *Alternaria* emerging
537 mycotoxins (AOH, AME, MAC, TEN, BEAU, DAS, EQUUS and MAS) mean levels recorded
538 in the current study were significantly higher in grains from north coastal area ($p < 0.01$). The
539 difference in mycotoxins contamination within different agroecological areas was expected
540 since the climate change from the north to the south of Tunisia, from humid to arid regions.
541 Furthermore, the genetic diversity of the Tunisian pearl millet across different agroecological
542 zones could probably interfere on its vulnerability to mycotoxins infestation within regions
543 (Ben Romdhane et al., 2018).

544 The inferred outcomes highlighted a high incidence of *Aspergillus* mycotoxins, namely
545 AFB1 and OTA, in the coastal south and continental center zones which are endowed with a
546 hot and arid climate with a mean monthly temperature ranged of 29.2-30.9 °C and a moderate
547 relative humidity (59-65%) during harvest period. The heat waves and dryness in addition to
548 low moisture, lead to greater xerophilic fungal proliferation namely, *Aspergillus* genera. In
549 fact, optimum temperature for *Aspergillus* species growth and mycotoxins production ranged
550 of 25-35 °C (Paterson & Lima, 2011). Besides, the authors reported higher optimal
551 temperatures for aflatoxin producing fungi. In addition, among the different spoilage infesting
552 cereals grains, they have the ability to cope with low water activity (Lasram, Hamdi,
553 Chenenaoui, Mliki, & Ghorbel, 2016; Parra & Magan, 2004). Thus, *Aspergillus* species will
554 tend to outcompete other fungi less liable to hot climate and rough environmental conditions.
555 Consequently, in arid sampling zones, aflatoxins and ochratoxins have been alluded to
556 dominate other fungal secondary metabolites produced by *Alternaria* and *Fusarium* which
557 are, generally, more adapted to lower temperatures with optimal growth temperatures in the
558 ranges of 20-25 and 15-30 °C, respectively (Paterson & Lima, 2011). Accordingly, in the
559 north coastal zone, AFB1 and OTA have been supplanted by *Alternaria* and *Fusarium*
560 metabolites (AOH, AME, MAC, TEN, BEAU, DAS, EQUUS and MAS). In fact, in Tunisia,
561 the prevailing sub-humid climate in the north coastal zone, characterized by a mild and wet
562 weather in addition to a warm temperature, with a mean monthly temperature of about 26°C
563 and a high relative humidity (68-75%) during the harvest period. Several reports showed that
564 fungal geographic worldwide distribution and mycotoxins patterns have a strong reliance on
565 climate change and environmental conditions, mainly temperature, humidity and precipitation
566 (Abia, et al., 2013; Adetunji, et al., 2014; Lasram, et al., 2012; Ojochenemi, et al., 2016;
567 Oyedele, et al., 2017; Van der Fels-Klerx, Liu, & Battilani, 2016; Wang, et al., 2018; Xu,
568 Han, & Li, 2019).

569 Overall, the overlap environmental conditions corresponding to the climate factors,
570 harvesting periods, poor storage and transport conditions, wrong handling and agriculture
571 practices might trigger fungal proliferation and exacerbate mycotoxins production in Tunisian
572 pearl millet. Additionally, grain damage mainly due to insects invasion, might induce greater
573 fungal ingress and mycotoxin production. Thus, it's critical to increase awareness by
574 implementing preventive strategies, proper and adequate agriculture practices that would
575 mitigate mycotoxins issue in food and feed commodities especially, that in Tunisia, pearl
576 millet crop is usually cultivated in harsh conditions by smallholder subsistence farmers.

577 Mycotoxins risk assessment in Tunisian pearl millet revealed, a worrisome situation that
578 have to be faced by setting up strenuous regulatory thresholds and a strict control system
579 within the food and feed trade, in order to prevent and narrow mycotoxins as a major issue
580 requiring priority attention.

581 Furthermore, the current regulations mostly take account about major mycotoxins namely
582 (AFs, OTA, DON, ZEA, FBs) and scarcely about emerging mycotoxins and derivatives
583 produced by several fungi occurring in food and feed. Consequently, the undeniable
584 toxicological effects on human and animals health associated to a mixture of toxic metabolites
585 exposure, highlighted the obvious challenge to widen the legislations in order to encompass
586 further mycotoxins with respect to the food consumption patterns. Noteworthy, Tunisia is
587 following the European mycotoxins legislations. Thus, we suggest in our study to establish
588 specific regulatory thresholds to pre-empt the food safety risks and to promote a better
589 position in the international trade.

590 In conclusion, the current work asserts for the first time in north African countries, an
591 overview of regulatory and emerging mycotoxins occurrence in the Tunisian pearl millet,
592 intended for human consumption. Further studies are required to investigate the contribution

593 of pearl millet in the daily intake of mycotoxins by Tunisian consumer for the monitoring of
594 the risk assessment.

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Fig 1. Sampling locations of Pearl millet (*P.glaucum*) in Tunisia

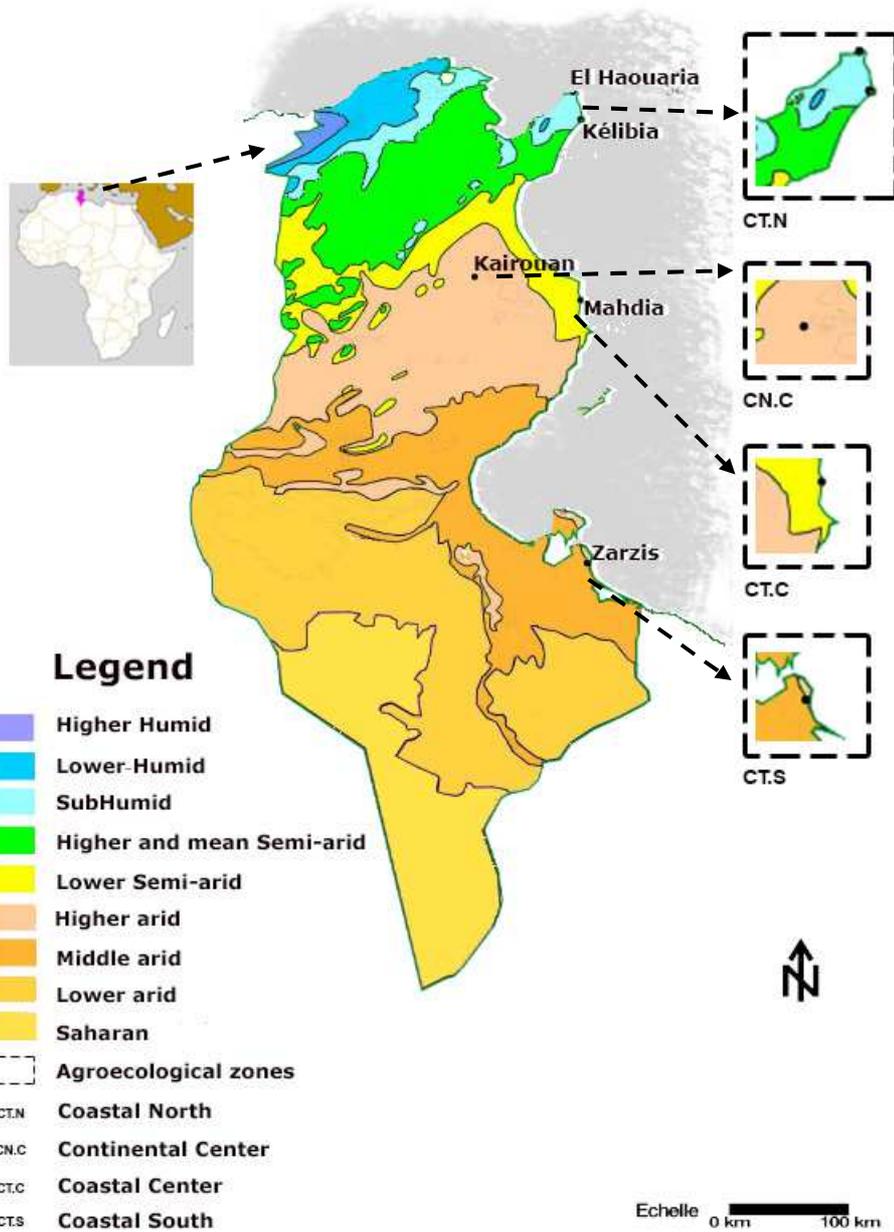


Table 1. Sampling provenance and bioclimatic characteristics of different sampling agroecological zones (AGZ).

Sample provenance	AGZ	Sample number	Annual precipitation (mm/year)	Mean monthly temperature (°C)
Farmers (n=144)	CT.N	69	500 – 700	5.5 – 35.3
	CT.C	10	300 – 400	7.7 – 31
	CN.C	45	200 – 300	5 – 37.1
	CT.S	10	100 – 200	6.9 – 34.3
Commerce (n=76)	–	76	–	–

Table 2. Major mycotoxins and derivatives present in pearl millet from Tunisia (n = 220).

Metabolites	LOD (µg/kg)	Farmers samples (n=144)			Total commercial samples (n=76)				Total millet samples (n=220)				
		PS (%)	Concentration (µg/kg) of positive samples			PS (%)	Concentration (µg/kg) of positive samples			PS (%)	Concentration (µg/kg) of positive samples		
			Mean	Median	Max		Mean	Median	Max		Mean	Median	Max
Major mycotoxins and derivatives													
Aflatoxin B1	0.24	6.3	181	36.6	1046	13.2	39.4	20.8	117	8.6	106	29.6	1046
Aflatoxin B2	0.4	0.7	96.1	96.1	96.1	0	0	0	0	0.5	96.1	96.1	96.1
Aflatoxin G1	0.32	0	0	0	0	1.3	20.3	20.3	20.3	0.5	20.3	20.3	20.3
Aflatoxin M1	0.4	0.7	18.1	18.1	18.1	0	0	0	0	0.5	18.1	18.1	18.1
Ochratoxin A	0.4	2.8	102	97	178	19.7	60.6	31.8	231	8.6	69.4	36	231
Ochratoxin B	8	0	0	0	0	1.3	52.1	52.1	52.1	0.5	52.1	52.1	52.1
Zearalenone	0.12	0.7	13.1	13.1	13.1	15.8	16.3	10.6	40.2	5.9	16	12.2	40.2
Zearalenone-sulfate	1.6	0.7	425	425	425	0	0	0	0	0.5	425	425	425
Nivalenol	1.2	0	0	0	0	2.6	114	114	161	0.9	114	114	161
Fusarium metabolites													
Monoacetoxyscirpenol	1.6	16	130	82.4	339	2.6	82.8	82.8	113	11.4	126	82.4	339
Diacetoxyscirpenol	0.4	15.3	63.1	47.7	168	1.3	27.3	27.3	27.3	10.5	61.5	46.2	168
Enniatin A2	0.032	0.7	0.78	0.78	0.78	0	0	0	0	0.5	0.78	0.78	0.78
Enniatin B	0.024	1.4	0.75	0.75	0.97	2.6	2.41	2.41	4.38	1.8	1.58	0.75	4.38
Enniatin B2	0.04	0.7	0.71	0.71	0.71	0	0	0	0	0.5	0.71	0.71	0.71
Beauvericin	0.008	45.1	0.82	0.39	13	39.5	1	0.72	5	43.2	0.87	0.45	13
Equisetin	0.24	47.9	158	44.7	1909	34.2	99.8	49.8	588	43.2	142	49.2	1909
Epiequisetin	0.24	4.9	10	8.67	21.1	2.6	16.7	16.7	27.3	4.1	11.5	8.67	27.3
Chrysogin	0.4	2.1	49.8	45.3	77.2	22.4	53.4	46.9	102	9.1	52.8	46.1	102
Deoxyfusapyron	0.8	1.4	18.3	18.3	18.9	23.7	36.1	33.4	83.5	9.1	34.3	32.2	83.5
Fusapyron	0.8	2.1	69.3	68.4	118	31.6	119	105	376	12.3	113	98.5	376
Alternaria metabolites													
Alternariol	0.4	11.8	59.4	28.2	194	14.5	40.9	25.6	130	12.7	52.1	26.9	194
Alternariolmethylether	0.032	45.1	78.7	14.5	777	61.8	115	14.5	1351	50.9	94.2	14.5	1351
Tentoxin	0.08	64.6	14.4	7.6	111	31.6	14.6	10.3	54.7	53.2	14.5	8.2	111
Tenuazonic acid	8	14.6	125	113	505	13.2	39.9	31.9	94.5	14.1	98.1	52.8	505
Macrosporin	0.04	52.8	94	22.3	2302	36.8	84.8	72	437	47.3	91.5	37.2	2302
Altersolanol	24	15.3	4268	2541	22005	5.3	1034	1122	1491	11.8	3770	2052	22005
Altersetin	0.4	18.8	32.9	9.35	339	0	0	0	0	12.3	32.9	9.35	339
Infectopyron	8	1.4	318	318	363	0	0	0	0	0.9	318	318	363
Aflatoxin precursors													
Sterigmatocystin	0.32	0.7	15.1	15.1	15.1	5.3	48.8	47.9	86.7	2.3	42.1	20.4	86.7
Averantin	0.04	2.1	7.55	6.24	11.4	2.6	4.6	4.6	5.24	2.3	6.37	5.24	11.4
Averufin	0.064	7.6	12.8	6	42.5	7.9	10.4	10.4	16.3	7.7	12	6.71	42.5
Versicolorin A	0.24	1.4	48.5	48.5	81.9	0	0	0	0	0.9	48.5	48.5	81.9
Versicolorin C	0.24	0.7	10.5	10.5	10.5	0	0	0	0	0.5	10.5	10.5	10.5
Other Aspergillus metabolites													
3-Nitropropionic acid	0.8	0.7	815	815	815	9.2	505	238	1476	3.6	544	346	1476
Kojic acid	16	9.7	9021	2662	41649	3.9	3187	2576	4571	7.7	7992	2576	41649
Malformin A	0.8	0.7	66	66	66	0	0	0	0	0.5	66	66	66
Malformin C	0.16	0.7	7.57	7.57	7.57	0	0	0	0	0.5	7.57	7.57	7.57
Agroclavine	0.24	1.4	387	387	712	27.6	1446	60.7	15108	10.5	1354	61.59	15108

Table 1. (Continued)

Metabolites	LOD (µg/kg)	Farmers samples (n=144)				Total commercial samples (n=76)				Total millet samples (n=220)			
		PS (%)	Concentration (µg/kg) of positive samples			PS (%)	Concentration (µg/kg) of positive samples			PS (%)	Concentration (µg/kg) of positive samples		
			Mean	Median	Max		Mean	Median	Max		Mean	Median	Max
Penicillium metabolites													
Curvularin	0.8	4.2	64.2	39.4	200	3.9	65	77.8	88.6	4.1	64.5	49.7	200
Oxaline	0.4	5.6	21.3	21.9	26.1	0	0	0	0	3.6	21.3	21.9	26.1
Atpenin A6	0.056	0.7	4.31	4.31	4.31	0	0	0	0	0.5	4.31	4.31	4.31
Citreorosein	2	1.4	33.9	33.9	42.9	1.3	27.5	27.5	27.5	1.4	31.8	27.5	42.9
Dihydrocitrinone	2	0.7	2851	2851	2851	0	0	0	0	0.5	2851	2851	2851
Citrinin	0.16	0	0	0	0	6.6	6921	2446	25663	2.3	6921	2446	25663
Quinolactacin A	0.08	2.8	6.36	5.94	9.07	0	0	0	0	1.8	6.36	5.94	9.07
Bacterial metabolites													
Chloramphenicol	0.12	1.4	19.17	19.17	35.6	0	0	0	0	0.9	19.17	19.17	35.6
Ascochlorin	0.16	2.8	14.43	6.41	39.91	1.3	8.28	8.28	8.28	2.3	13.2	7.02	39.91
Antibiotic PF 1053	1.6	0	0	0	0	1.3	11.9	11.9	11.9	0.5	11.9	11.9	11.9
Metabolites from unspecified sources													
Asperglaucide	0.08	16.7	141	7.8	1275	76.3	51.4	15.2	477	37.3	77.6	13.8	1275
cyclo(L-Pro-L-Tyr)	0.8	4.9	44.6	41.9	56	18.4	57.9	57.2	92.3	9.5	53.5	54.3	92.3
Xanthotoxin	0.32	6.3	159.3	62.6	802	1.3	234	234	234	4.5	167	76.5	802
Monocerin	0.4	43.1	166	113	805	55.3	395	135	2142	47.3	259	118	2142
Emodin	0.056	6.9	5.83	3.58	26.2	26.3	6.01	4.99	16.9	13.6	5.95	4.19	26.2
Asperphenamate	0.04	8.3	16.6	5.26	91.8	0	0	0	0	5.5	16.6	5.26	91.8
Neoechinulin A	0.64	2.1	90.8	40.6	196	5.3	71.6	64.7	110	3.2	79.8	61.9	196
Usnic acid	0.032	0.7	0.51	0.51	0.51	2.6	1.7	1.7	2.12	1.4	1.3	1.27	2.12
Lotaustralin	0.32	0	0	0	0	1.3	22.7	22.7	22.7	0.5	22.7	22.7	22.7

Table 3. Summary of significant mycotoxins and metabolites in Pearl millet across the different agro-ecological zones of Tunisia. **a.** *Aspergillus* metabolites (AB1=Aflatoxin B1; OTA= Ochratoxin A; KA= Kojic acid); **b.** *Fusarium* metabolites (BEAU= Beauvericin; DAS= Diacetoxyscirpenol; EQUUS= Equisetin; MAS= Monoacetoxyscirpenol); **c.** *Alternaria* metabolites (AOH= Alternariol; AME= Alternariolmethylether; MAC= Macrosporin; TEN=Tentoxin).

a.

AGZ	Parameters	<i>Aspergillus</i> metabolites		
		AFB1	OTA	KA
CT.N (n=69)	PS (%)	1.4	0	2.9
	Range (µg/kg)	0 - 36.6	0	1722 - 1906
	Mean ±SD	0.53 ±4.41 ^b	<LOD ^a	52.6 ±307 ^b
CT.C (n=10)	PS (%)	0	0	0
	Range (µg/kg)	<LOD	<LOD	<LOD
	Mean ±SD	<LOD ^b	<LOD ^a	<LOD ^b
CT.S (n=20)	PS (%)	15	5	5
	Range (µg/kg)	12.2 - 1046	0 - 139	0 - 3375
	Mean ±SD	57.8 ±233 ^a	7 ±31.28 ^a	168 ±754 ^b
CN.C (n=45)	PS (%)	11.1	6.7	24.4
	Range (µg/kg)	18.4 - 244	35.5 - 178	1496 - 41649
	Mean ±SD	9.68 ±40.2 ^a	5.97 ±28 ^a	2651 ±8744 ^a
	p value (KW test)	0,048 *	0,164 ^{ns}	0,001 **

b.

AGZ	Parameters	<i>Fusarium</i> metabolites			
		BEAU	DAS	EQUS	MAS
CT.N (n=69)	PS (%)	69.6	31.9	78.3	33.3
	Range (µg/kg)	0.09 - 13	23.2 - 168	3.16 - 1909	47.2 - 339
	Mean ±SD	0.65 ± 1.72 ^a	20.1 ± 37.4 ^a	149 ± 310 ^a	43.4 ± 80 ^a
CT.C (n=10)	PS (%)	30	0	10	0
	Range (µg/kg)	0.32 - 0.65	<LOD	0 - 4.79	<LOD
	Mean ±SD	0.13 ± 0.23 ^b	<LOD ^b	0.48 ± 1.51 ^b	<LOD ^b
CT.S (n=20)	PS (%)	5	0	0	0
	Range (µg/kg)	0 - 0.34	<LOD	<LOD	<LOD
	Mean ±SD	0.02 ± 0.08 ^b	<LOD ^b	<LOD ^b	<LOD ^b
CN.C (n=45)	PS (%)	28.9	0	31.1	0
	Range (µg/kg)	0.09 - 1.16	<LOD	3.87 - 145	<LOD
	Mean ±SD	0.14 ± 0.29 ^b	<LOD ^b	13.2 ± 33.9 ^b	<LOD ^b
	p value (KW test)	< 0,0001 ***	< 0,0001 ***	< 0,0001 ***	< 0,0001 ***

c.

AGZ	Parameters	<i>Alternaria</i> metabolites			
		AOH	AME	Macrosporin	TEN
CT.N (n=69)	PS (%)	21.7	69.6	87	88.4
	Range (µg/kg)	15 - 194	0.85 - 777	0.96 - 2302	2.1 - 111
	Mean ±SD	12.9 ±37.2 ^a	66.8 ±165 ^a	93.3 ±301 ^a	16.6 ±20.8 ^a
CT.C (n=10)	PS (%)	0	0	50	50
	Range (µg/kg)	<LOD	<LOD	1.53 - 125	3.66 - 16
	Mean ±SD	<LOD ^b	<LOD ^b	18.87 ±40.5 ^b	4.61 ±6.16 ^b
CT.S (n=20)	PS (%)	0	10	35	45
	Range (µg/kg)	0	3.33 - 92.2	1.37 - 121	2.84 - 16
	Mean ±SD	<LOD ^b	4.78 ±20.6 ^b	19.4 ±37.7 ^b	2.89 ±4.5 ^b
CN.C (n=45)	PS (%)	4.4	33.3	8.9	40
	Range (µg/kg)	28.2 - 91.5	1.43 - 140	1.08 - 123	2.23 - 14.6
	Mean ±SD	2.66 ±14.1 ^b	9.04 ±26.2 ^b	2.83 ±18.4 ^c	2.05 ±3.42 ^b
	p value (KW test)	0,006 **	< 0,0001 ***	< 0,0001 ***	< 0,0001 ***

CT.N: Coastal north; CT.C: Coastal center; CT.S: Coastal south; CN.C: Continental center
ns: non-significant

* p<0.05

** p<0.01

*** p< 0.001