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

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Abstract

The current study assesses the natural occurrence of mycotoxins and fungal
metabolites in Tunisian Pearl millet. Investigations using a multi-mycotoxin liquid
chromatography/electrospray ionization-tandem mass spectrometric (LC/ESI-MS/MS)
method were carried out on a total of 220 pearl millet samples collected from farmers (n=144)
located in four different agroecological zones in Tunisia and from retail shops (n=76). Results

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

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

1 Introduction

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

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

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

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

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

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

2 Materials and methods

2.1 Sampling

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

2.2 Mycotoxins analysis

2.2.1 Chemicals and Reagents

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

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

2.2.2 Extraction and matrix effect

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

2.2.3 LC/ MS-MS parameters

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

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

2.3 Statistical analysis

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

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

3 Results

3.1 Major mycotoxins

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

Among major mycotoxins, aflatoxins, namely aflatoxin B1 (AFB1) were the most prevalent. Out of 10% of pearl millet samples contaminated with aflatoxins (22/220), 86.4% (19/22) contained AFB1 at an average of 106 $\mu\text{g/kg}$ and a median of 29.6 $\mu\text{g/kg}$ in positive millet samples. Taking into consideration all samples, the mean contamination of millet with AFB1 was about 9.2 $\mu\text{g/kg}$. 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 $\mu\text{g/kg}$ mean and 1046 $\mu\text{g/kg}$ maximum) comparing to the CT.N (0.53 $\mu\text{g/kg}$ mean and 36.6
225 $\mu\text{g/kg}$ maximum) and CN.C zones (9.67 $\mu\text{g/kg}$ mean and 245 $\mu\text{g/kg}$ 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 $\mu\text{g/kg}$, 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 $\mu\text{g/kg}$ (117 $\mu\text{g/kg}$ maximum) and 181 $\mu\text{g/kg}$ (1046 $\mu\text{g/kg}$ 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 $\mu\text{g/kg}$. Aflatoxin G1 (AFG1) was also
234 detected in only one commercial sample with an amount of 20.3 $\mu\text{g/kg}$.

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 $\mu\text{g/kg}$ and 36 $\mu\text{g/kg}$ median. With respect to
237 negative samples, the average level was 6 $\mu\text{g/kg}$. 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 $\mu\text{g/kg}$, respectively. In fact, OTA was observed in 19.7% of commercial samples
243 with mean amount of 60.6 $\mu\text{g/kg}$ (231 $\mu\text{g/kg}$ maximum) for positives samples, while, in
244 farmers' samples, OTA was detected at low frequency (2.8%) with high levels (102 $\mu\text{g/kg}$
245 mean, 178 $\mu\text{g/kg}$ maximum of positives samples).

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

3.2 Emerging metabolites

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

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

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

Alternaria metabolites occurred with an incidence reaching 13.8% out of the total 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

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

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

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

4 Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Declarations of interest: none

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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; EQU= 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