Arabian Journal of Chemistry (2020) 13, 6753–6761

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Mycotoxins presence in pre- and post-fermented silage from Tunisia

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Received 10 April 2020; accepted 21 June 2020
Available online 26 June 2020

KEYWORDS

Silage;
Post-fermented;
Pre-fermented;
Mycotoxin;
LC-MS/MS;
QuEChERS;
Tunisia

Abstract Silage represents a major part of the feed ration for livestock, being a potential cause of mycotoxicosis as it can be contaminated with toxigenic fungi capable of producing mycotoxins in suitable environmental conditions.

In the present work, the presence of natural mycotoxins in samples collected of silage from the main producing areas in Tunisia (Ariana, Bizerte, Béjà and Jendouba) was assessed based on different plant material (oat, barley, triticale, artichoke, sulla or raygrass). Mycotoxins were evaluated during three periods in green forage (P1) and subsequently in ensiled products after 60 days (P2) and 100 days (P3) of fermentation. Samples were extracted by a QuEChERS procedure and analyzed by mass spectrometry for the determination of 23 mycotoxins. The results showed the presence of *Fusarium* mycotoxins, deoxynivalenol (DON), HT-2 toxin, zearalenone (ZEA), enniatins (ENA1, ENB, ENB1) and beauvericine (BEA); as well as one *Alternaria* mycotoxin tentoxin (TENT). The highest values detected were for DON in the three periods. DON maximum value were 381, 2053 and 916 μg/kg for P1, P2 and P3, respectively. This study demonstrated the presence of fungi and DON, ZEA and ENs contamination in Tunisian silage without a recognized risk on the ruminants or further in humans. However, a continuous enhancement of the silage quality, and management and control of mycotoxins, should be implemented to ensure safe ensilable plant material.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.arabjc.2020.06.029
1878-5352

1. Introduction

The animal feed system in Tunisia is experiencing numerous disturbances related to climatic, economic, social and environmental factors (Ben Salem et al., 2011). This could be explained by irregular and seasonal distribution of feed production as well as the extensive characteristics of livestock farming.

Nowadays, the main issues encountred in livestock farming in Tunisia are related to a significant decrease in rangelands, a considerable drop of the consumable biomass yield as well as the lack of fodder which remains of poor quality (Jemaa et al., 2016; Ben Salem et al., 2011; Mohamed-Brahmi, Khaldi & Khaldi, 2010).

The availability of stored food (wet or dry) during periods of shortages is a crucial solution to overcome the animal weight losses and avoid a potential mortality (Olivier et al., 2015). Silage is a technique for preserving green fodder through lactic fermentation under anaerobic conditions. This fermentation allows the stabilization of the plant material by minimizing losses of dry matter and nutritional value as well as the inhibition of the growth of undesirable microorganisms due to pH value drops (4–4.5) (del Palacio et al., 2016) and the effect of lactic acid towards other microorganisms (Ma et al., 2017). However, under several climatic conditions, presence of water, relative humidity, temperature or inadequate storage practices, allows molds growing and production of harmeful secondary metabolites as mycotoxins, may affect the silage safety (Boudra, 2009).

Furthermore, the silage production is not standerized, so that the silage quality and consequently the mycotoxins levels may vary widely due to various factors such as an inadequate rate of dry matter during harvest, a slow filling of the silo, a bad settlement, losses during anaerobic storage, imperfect hermeticity of the silo and aerobic deterioration after opening the silo (Gallo et al, 2015).

Mycotoxins are secondary metabolites produced by toxicogenic fungi (*Aspergillus, Penicillium, Alternaria* and *Fusarium*) in order to increase its competitiveness front other microorganisms and to increase substrates available for its growth (Duarte et al., 2011; Yiannikouris & Jouany, 2002). Mycotoxins can be developed on the plant in the field during its growth, in pasture or during storage (hay, in silage) during poor storage or handling conditions. *Fusarium* fungal genera can lead not only to mycotoxins contamination but to alter organoleptic and nutritive qualities of stored foods leading to a decrease in zootechnical performance (milk production, body weight gain, reproduction …) (Akande et al., 2006). Consumer can be exposed to mycotoxins throught meat or other animal derived products such as eggs or milk (Duarte, Lino & Pena, 2011). There is currently an important data on mycotoxins in animal nutrition. However, little work has been undertaken to study the adverse effects of mycotoxins on the quality of livestock feed.

In Tunisia, there is not available data on the effects of toxigenic fungi and mycotoxins on the quality of feed and the ruminants performances. To the better of our knowledge, this is the first report ever on silage feedstuffs performed in Tunisian samples. The present work aim to evaluate the natural muti-mycotoxins presence in green forage and subsequent ensiling products after 60 and 100 days fermentation within the optic of a better understanding of the mycotoxins dynamics and the conditions leading to follow up their toxicity problems in ruminants.

2. Materials and methods

2.1. Chemicals and reagents

Solvents (acetonitrile, hexane and methanol) were purchased from Merck KGaA (Darmstadt, Germany). Anhydrous magnesium sulphate was obtained from Alfa Aesar GmbH & Co. (Karlsruhe, Germany); sodium chloride was purchased from Merck and C18 was purchased from Phenomenex (Torrance, USA). The derivatization reagent composed of BSA (N,O-bis(trimethylsilyl) acetamide), TMCS (trimethylchlorosilane) and TMSI (N-trimethylsilylimidazole) were purchased from Supelco (Bellefonte, PA). Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain). The standards of aflatoxins (AFB1, AFB2, AFG1, AFG2), ochratoxin A (OTA), sterigmatocystine (STG), alternariol monomethylether (AME), alternariol (AOH), tentoxin (TENT), zearalenone (ZEA), nivalenol (NIV), deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3AcDON), 15-acetyl-deoxynivalenol (15AcDON), diacetoxyscirpenol (DAS), neosolaniol (NEO), T-2, HT-2 toxin, beauvericine (BEA), enniatins (ENA, ENA1, ENB and ENB1), were purchased from Sigma Aldrich (Madrid, Spain).

2.2. Sampling

A set of feed samples (n = 84) were collected for the present survey. Fresh grass and consequent silages were taken from different silos where intensive breeding and silage production are practiced, and located in four regions: in Ariana and Bizerte located in Tunisian Northen area and Béjà and Jendouba in the Tunisian North West area. Samples were mainly composed of cereals (oat, barley, tritical) as well as other adjuvant plant material such as artichoke, sulla or ryegrass. Sampling was carried out from April to September 2018. According to the period of fermentation, three different set of samples were collected from each studied silo: i) before compaction (P1), ii) 60 days after (P2) fermentation starts but the material is not used in the feeding process yet; and iii) after 100 days of compaction (P3) when the fermentation is stopped and silage can be used. The sampling procedure is detailed as follows: At P1 stage, fresh material was taken randomly during silo filling; while, at P2 and P3 stages, plant material was collected from three different layer of the silos (upper, middle and lower) at 50 cm depth. Plant material was homogenized and mixed thoroughly to obtain a representative sample of 1 kg placed in sealed plastic bags. Samples were dried at 50 °C for 48 h, milled into fine powder and stored at −20 °C in PTFE tubes prior to the analysis for mycotoxins presence.

2.3. Mycotoxin analysis

2.3.1. QuEChER extraction

Tweenty three mycotoxins were extracted according to the methodology developed by Juan et al., (2017) with few

modifications. The parameters of instrumental linearity, matrix effect, sensitivity, accuracy and precision were studied for validation method, according to the EU Commission Decision, 2002/657/EC (EC, 2002). These validation was done previously to apply on studied sample.

Briefly, 5 g of each homogenized sample were extracted with 30 mL of acetonitrile/water /acetic acid (79:20:1, v/v/v) homogenized for 15 min in an horizantal shaker and centrifuged for 5 min at 1792*g* (G-force) and 5 °C using an Eppendorf Centrifuge 5810R (Eppendorf, Hamburg, Germany).

The upper layer was filtered through Whatman filter paper and transfered into 50 mL centrifuge tube. Then, 4 g of $MgSO_4$ and 1 g of NaCl were added. The mixture was shaken for 2 min and centrifuged (10 min, 1792*g*, 5 °C). Afterwards, 2 mL of the upper-layer was transferred into 15 mL centrifuge tube and added 100 mg of C18 and 600 mg of $MgSO_4$. The tube was shaken for 1 min and centrifuged for 10 min at 1792*g* and 5 °C. Finally, the upper layer was filtered through a syringe nylon filter (13 mm/0.22 μm) purchased from Anàlisis Vínicos S.L (Tomelloso, Spain). The final volume was transfered into conical vials, 250 μL for derivatization and GC–MS/MS analysis and the rest for LC-MS/MS analysis.

2.3.2. Derivatization

Before GC–MS/MS analysis, 250 μL of the filtred were dried under a gentle stream of nitrogen using a Zymark TurboVap LV Evaporator. The dry extracts were re-dissolved in 50 μL of BSA + TMCS + TMSL (3:2:3) and left for 30 min to react at room temperature. The derivatized sample was diluted to 250 μL with hexane and vortexed for 30 s. The derivatized was purified with a liquid–liquid extraction adding 1 mL of phosphate buffer (60 mM, pH 7). Finally, 100 μL of the upper layer was transferred to an autosampler vial for its GC analysis.

2.3.3. GC–MS/MS analysis

The final extract was injected in splitless mode at 250 °C in programmable temperature vaporization (PTV) using an Agilent 7890A GC system coupled with an Agilent 7000A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, CA). The mass spectrometer was operating in electron impact ionization (EI, 70 eV). The source and transfer line temperatures were 230 °C and 280 °C, respectively. The collision gas for MS/MS experiments was nitrogen, and the helium was used as carrier gas at fixed pressure of 20.3 psi, both at 99.999% purity supplied by Carburos Metálicos S. L. (Barcelona, Spain). Data have been acquired and processed using the Agilent MassHunter version B.04.00 software. The quantification and confirmation transition were performed as described previously by Oueslati et al., (2018). Eigh mycotoxins were analyzed by GC–MS/MS. NIV, DON, 3-ADON, 15-ADON, DAS, NEO, T-2 and HT-2 and have been separated on a HP-5MS (30 m × 0.25 mm × 0.25 μm) capillary column (Sup. Figure 1). The oven temperature program was initially 80 °C, and the temperature increased to 245 °C progressively at 60 °C/min. After a 3 min hold time, the temperature was increased to 260 °C progressively at 3 °C/min and finally to 270 °C at 10 °C/min and then held for 10 min.

2.3.4. LC–MS/MS analysis

From filtered final volum, as indicate in Section 2.3.1., 0.5 mL was evaporated to dryness at 38 °C under a gentle stream of nitrogen using a multi-sample Turbo-vap LV Evaporator (Zymark, Hoptkinton, USA). Before LC-MS/MS analysis the dry residue was reconstituted to a final volume of 0.5 mL with methanol/water (70:30, v/v) and filtered through a 13 mm/0.22 μm nylon filter purchased from Anàlisis Vínicos S.L (Tomelloso, Spain).

The analysis was performed using a LC Agilent 1200 using a binary pump with automatic injector, and coupled to a 3200 QTRAP® AB SCIEX (Applied Biosystems, Foster City, CA) equipped with a Turbo-VTM source (ESI) interface. The chromatographic separation of the analytes was conducted at 25 °C with a reverse phase analytical column Gemini®NX-C18 (3 μm, 150 × 2 mm ID) and a guard column C18 (4 × 2 mm, ID; 3 μm) from Phenomenex (Madrid, Spain). Mobile phase was a time programmed gradient using methanol as phase A (0.1% formic acid and 5 mM ammonium formate), and water as phase B (0.1% formic acid and 5 mM ammonium formate). The following gradient was used: equilibration for 2 min at 90% B, decrease linearly to 20% of phase B in 3 min, maintain 20% of phase B for 1 min, decrease linearly from 20 to 10% of phase B in 2 min, maintain 10% of phase B for 6 min, decrease to 0% B in 3 min, maintain 100% A for 1 min, finally increase linearly from 0 to 50% B in 3 min, return to initial conditions (90% B) in 2 min and maintain during 2 min. The flow rate was 0.25 mL/min in all steps. Total run time was 21 min. The injection volume was 20 μL.

With regard to mycotoxin analysis, the QTRAP System was used as triple quadrupole mass spectrometry detector (MS/MS) (Sup Fig. 2 and Fig. 3). The Turbo-V™ source was used in positive mode to analyze the 15 mycotoxins (AFs, OTA, STG, AME, AOH, TENT, ZON, ENs and BEA) with the following settings for Source/Gas Parameters: Vacuum Gauge (10e−5 Torr) 3.1, curtain gas (CUR) 20, ionspray voltage (IS) 5500, source temperature (TEM) 450 °C, ion source gas 1 (GS1) and ion source gas 2 (GS2) 50. The precursor ions (Q1), product ions (Q3), collision energies (CE), collision cell exit potential (CXP), declustering potential (DP) and collision cell entrance potential (CEP) (Stanciu et al., 2017). The entrance potential (EP) was the same for all analytes, 10 V. Acquisition and processing data were performed using Analyst® software, version 1.5.2.

3. Results and discussion

Many regions in Tunisia, especially in the northern area, ensiled forages are highly valuable as animal feed used in major dairy farms. In order to assess the quality of silage from Tunisia, the present work evaluated the natural presence of 23 different mycotoxins during all the production process. The analytical method used in this investigation was based on both LC-MS/MS and GC–MS/MS techniques, which LOD and limit of quantification (LOQ) were estimated using an extract of the blank for a signal-to-noise ratio (S/N) $\geq$3 and $\geq$10, respectively, from chromatograms of samples spiked at the lowest level validated. Sensibility of which was below the European recommended levels, the limit of detection (LOD) values

Table 1 Sensibility (limit of detection and limit of quantification), of analyzed mycotoxins during the three studied stages.

Mycotoxin	LOD (μg/Kg)	LOQ (μg/Kg)	Recommendation (μg/Kg)
DON	1.31	4.38	900–12000
15-ADON	1.12	3.73	Rec. 2006/576/EC
3-ADON	1.86	6.21	
NIV	1.55	5.15	.
NEO	2.17	4.63	.
DAS	1.70	5.66	.
T-2	4.36	8.53	250–2000
HT-2	1.97	6.55	Rec. 2013/165/EU
ZEA	1.31	4.38	900–12000
			Rec. 2006/576/EC
ENA	0.51	1.70	–
ENA1	0.51	1.68	–
ENB	0.29	0.98	–
ENB1	0.48	1.60	–
BEA	0.38	1.27	–
AFB1	1.68	5.60	5–20*
AFB2	1.84	6.13	Directive 2003/100/EC
AFG1	1.59	5.29	
AFG2	2.09	7.64	
STG	2.6	5.2	–
AME	16.43	54.78	–
AOH	24.94	83.14	–
TENT	1.84	4.47	–
OTA	0.84	2.81	50–250
			Rec. 2006/576/EC

LOD:Limit of detection; LOQ: limit of quantification.

ranged from 0.5 to 25 μg/kg (Table 1). The validation results indicates that linear regression coefficients of all calibration curves demonstrated a good linearity, with corresponding correlation coefficients (r^2) higher than 0.9989. LDs and LQs of the mycotoxins analyzed presented a high variability and were between 0.1 and 500 μg/kg. The accuracy was evaluated for each compound and recovery (n = 6) values were between (67 ± 7) % (ZEA in Triticale) and (108 ± 9) % (BEA in Rayegrass). Regarding precision values evaluated from the relative standard deviation (RSDR) of intraday precision (n = 6) and interday precision (n = 9), these were lower than 9% and 15%, respectively.

The analysis revealed the presence of eight mycotoxins amoung the 23 tested being mainly *Fusariu*m mycotoxins (DON, HT-2 toxin, ZEA, ENA1, ENB, ENB1 and BEA) and one *Alternaria* mycotoxin, TENT (Table 2). These results are in compliance with those reported from various studies Gonzales Pereyra et al., 2008; Richard et al., 2009. McElhinney et al. (2015) reported no AFs, OTA, fumonisins and T-2 toxin in silage samples from Ireland. Another study in Uruguay indicated that freshly harvested samples were not contaminated with AFs (del Palacio et al., 2016). Further, no AFs were reported in silage samples from Poland (Kosicki et al., 2016; Panasiuk et al., 2019). Such observation may be due to the *Aspergillus* and *Penicillium* sp. intolerance to anerobic conditions and an acid environment. These previous results suggested the stability of these substances during fermentation (Boudra & Morgavi, 2008), however, it is important to notice that these mycotoxins did not appeared in post-fermentation phase of our study coinciding with Pereyra et al. (2008).

The highest mycotoxin values detected in our samples were for DON at all three stages. DON's maximum value were 381, 2053 and 916 μg/kg for P1, P2 and P3, respectively. However, the most detected mycotoxin was ENB that was found in the 27% of samples at both P1 and P2, and in the 34% of samples at P3.

3.1. Occurrence of mycotoxins in the studied periods

During the first stage (P1), the sampled green forage intended for silage production was contaminated with three *Fusarium* mycotoxins: DON, ENB and BEA. ENB was the most frequently detected (27%) followed by DON (15%) and BEA (4%). DON was present in four samples with a maximum amount reaching 381 μg/kg, although it did not exceed the EU maximum guidance levels (EC, 2006). The maxium amouts of DON (198 μg/kg), and ENB (0.4 μg/kg) were found in samples from the region of Bizerte; while BEA (0.2 μg/kg) maximum level was found in Ariana region (Table 2). Both region are located in the Northern side of Tunisia characterized by having a humid climate, high levels of precipitations and average temperature of 26 °C.

During the second stage (P2), DON, ZEA, TENT, ENA1, ENB, ENB1 and BEA contaminated the silage samples being under a fermentation process for 60 days. ENB was the most frequently mycotoxin, detected in 7 samples (27%) with a mean value of 0.7 μg/kg. The highest concentration detected was for DON reaching 2053 μg/kg, which did not either exceed the EU guidance values (EC, 2006). Whereas, ZEA, TENT and BEA were detected at lower levels of 17, 30 and 0.14 μg/kg in 4%, 8% and 4% of analyzed samples, respectively. It is important to notice that the maximum amounts were found in samples from the Bizerte region where both annual relative humdity (73%) and mean temperature (18 °C) are suitable for the growth of *Fusarium* spp.

Furthermore, in the third stage (P3), where samples were under 100 days of fermentation showed to be contaminated with DON (28%), HT-2 (9%), ENA1 (3%), ENB (34%) and ENB1 (9%). It was observed that DON levels increased compared with the other stages, to reach a mean value of 1539 μg/kg; nonetheless, not even at this stage exceeded the EU guidance values (EC, 2006). In addition, ZEA and BEA decreased to traces levels. However, ENA1, ENB and ENB1 manteined their trend with mean values of 0.02, 0.2 and 0.07 μg/kg, repectively. The high incidence and maximum amounts of mycotoxins, even if the levels decreased, were also found in samples from the Bizerte region. In fact, this observation corroborate the effect of both warm temperature and high humidity for the fungal growth and subsequent mycotoxins production. In our case, it was subsequently supposed that both *Fusarium graminearum* and *Fusarium avenaceum* co-existed all over the silage production process even if they are quite affected by the fermentation procedure and anaerobiosis conditions.

DON has been previously described as one of the most common mycotoxin found in silage (Storm et al., 2008; Gallo et al., 2015; Cogan et al., 2016; Kosicki et al, 2016) present at different concentrations in pre- and post-fermentation samples as reported in the literature: in Lithuania in grass mixture silage at 1100 μg/kg; in The Netherlands, in maize silage at

Table 2 Incidence (I), frequency (F) and total mean (M ± SD) of detected mycotoxins during the three stages in the four Tunisian studied regions.

P1 (N = 26)	Mycotoxin	Ariana (N = 4)		Bizerte (N = 7)		Béjà (N = 10)		Jendouba (N = 5)		TOTAL		
		I (F,%)	M ± SD (μg/Kg)	I (F,%)	M ± SD (μg/Kg)	I (F,%)	M ± SD (μg/Kg)	I (F,%)	M ± SD (μg/Kg)	I(F, %)	Max(μg/Kg)	M ± SD (μg/Kg)
	DON	-	-	1 (14)	54.4 ± 144	3(30)	63.3 ± 105	-	-	4 (15)	381	39 ± 242
	ENB	1 (25)	0.01 ± 0.02	3 (43)	0.3 ± 0.51	2 (20)	0.028 ± 0.07	1 (20)	0.036 ± 0.08	7 (27)	0.4	0.035 ± 0.26
	BEA	1(25)	0.05 ± 0.1	-	-	-	-	-	-	1 (4)	0.2	0.008 ± 0.14
P2 (N = 26)	**Mycotoxin**	**Ariana (N = 4)**		**Bizerte (N = 8)**		**Béjà (N = 9)**		**Jendouba (N = 5)**		**TOTAL**		
		I (F,%)	**M ± SD (μg/Kg)**	**I (F,%)**	**M ± SD (μg/Kg)**	**I (F,%)**	**M ± SD (μg/Kg)**	**I (F,%)**	**M ± SD (μg/Kg)**	**I(F, %)**	**Max(μg/Kg)**	**M ± SD (μg/Kg)**
	DON	–	–	2 (25)	380 ± 599	1 (11)	129 ± 411.2	1 (20)	90 ± 201	4 (15)	2053	179 ± 1325
	ENA1	–	–	1 (13)	0.04 ± 0.11	–	–	–	–	1 (4)	0.32	0.01 ± 0.22
	ENB	1 (25)	0.075 ± 0.15	4 (50)	0.6 ± 0.82	2 (22)	0.096 ± 0.21	2 (40)	0.048 ± 0.08	9 (27)	2.6	0.24 ± 1.7
	ENB1	–	–	1 (25)	0.175 ± 0.5	–	–	–	–	1 (4)	1.4	0.054 ± 0.9
	BEA	1 (25)	0.035 ± 0.07	1 (25)	0.0125 ± 0.035	–	–	–	–	2 (8)	0.14	0.0092 ± 0.09
	ZEA	1 (25)	4.27 ± 8.5	–	–	–	–	–	–	1 (4)	17.1	0.66 ± 1.2
	TENT	1 (25)	7.4 ± 14.8	–	–	–	–	–	–	1 (4)	29.6	1.14 ± 20
P3 (N = 32)	**Mycotoxin**	**Ariana (N = 6)**		**Bizerte (N = 11)**		**Béjà (N = 9)**		**Jendouba (N = 6)**		**TOTAL**		
		I (F,%)	**M ± SD (μg/Kg)**	**I (F,%)**	**M ± SD (μg/Kg)**	**I (F,%)**	**M ± SD (μg/Kg)**	**I (F, %)**	**M ± SD (μg/Kg)**	**I(F, %)**	**Max(μg/Kg)**	**M ± SD (μg/Kg)**
	DON	3 (50)	593 ± 131	4 (36)	807.3 ± 48	–	–	2 (33)	537 ± 33	9 (28)	915.9	153.9 ± 538
	HT-2	–	–	3 (18)	12.55 ± 42	–	–	1 (17)	5.17 ± 10	3 (9)	116	5.3 ± 78
	ENA1	2 (17)	0.083 ± 0.12	–	–	–	–	–	–	2 (3)	0.5	0.016 ± 0.34
	ENB	2 (33)	0.167 ± 0.20	5 (45)	0.41 ± 0.65	2 (22)	0.067 ± 0.14	2 (33)	0.07 ± 0.11	11 (34)	3	0.20 ± 1.98
	ENB1	1 (17)	0.143 ± 0.35	2 (18)	0.13 ± 0.32	–	–	–	–	3 (9)	0.86	0.071 ± 0.56

Table 3 Incidence (I) and mean of detected mycotoxins in total samples according to the type of silage and sampling stage (P1, P2 and P3).

Stage	Oat			Tritical			Rayegrass			Artichoke		
	N	Mycotoxin (I)	Mean (μg/Kg)	N	Mycotoxin (I)	Mean (μg/Kg)	N	Mycotoxin (I)	Mean (μg/Kg)	N	Mycotoxin (I)	Mean (μg/Kg)
P1	10	–	–	5	DON (2)	558 ± 1517	2	ENB (1)	0.12 ± 0.11	3	DON (1)	832 ± 1266
		–	–		EN B (2)	0.8 ± 1.9		–	–		ENB (1)	0.067 ± 0.1
		–	–		BEA (1)	0.05 ± 0.1		–	–		–	–
		–	–		–	–		–	–		–	–
P2	15	DON (4)	310 ± 666	2	BEA (1)	0.05 ± 0.013	2	–	–	2	ENB (1)	0.15 ± 0.15
		EN A1 (1)	0.02 ± 0.4		ZEA (1)	8.55 ± 9.9		–	–		BEA (1)	0.07 ± 0.049
		ENB (7)	0.37 ± 0.8		–	–		–	–		TENT (1)	14.8 ± 18.9
		ENB1 (1)	0.093 ± 0.026		–	–		–	–		–	–
P3	15	DON (5)	429 ± 148	3	EN A1 (2)	0.1 ± 0.2	2	–	–	3	–	–
		ENB (4)	0.09 ± 0.89		EN B1 (2)	0.28 ± 0.072		–	–		–	–
		HT-2 (3)	7.71 ± 1.91		ENB (3)	0.05 ± 0.69		–	–		–	–
		ENB1 (1)	0.2 ± 0.052		DON (1)	245 ± 63		–	–		–	–
		BEA (1)	0.095 ± 0.067		–	–		–	–		–	–
Stage	Oat + Triticale			Oat + Barley			Sulla + Oat			Sulla + Rayegrass		
	N	Mycotoxin (I)	Mean (μg/Kg)	N	Mycotoxin (I)	Mean (μg/Kg)	N	Mycotoxin (I)	Mean (μg/Kg)	N	Mycotoinx (I)	Mean (μg/Kg)
P1	2	DON (2)	2309 ± 1632	2	ENB (1)	0.1 ± 0.1	2	ENB (2)	0.7 ± 0.18	1	–	–
P2	1	–	–	2	–	–	2	ENB (1)	0.32 ± 0.33	1	–	–
P3	2	DON (2)	412 ± 291.3	2	ENB (2)	0.04 ± 0.028	2	ENB (2)	0.5 ± 0.7	1	HT2 (1)	22.4
		–	–		–	–		DON (1)	925 ± 382.4		–	–

N: number of analyzed samples

Table 4 Co-occurrence of different mycotoxins: combinations of mycotoxins by periods and type of silage.

Number of Mycotoxins	P1		P2		P3	
	Combination	Type of silage	Combination	Type of silage	Combination	Type of silage
2 Mycotoxin	DON + ENB	Oat (1)	DON + ENB	Oat (2)	DON + HT-2	Triticale (1) Triticale + Oat (1)
	HT-2 + ENB	Oat (1)			DON + ENB	Oat (2) Sulla + Oat (2)
	ENB + ENB1	Oat (1)			ENB + ENB1	Oat (2) Triticale (1)
					ENB + BEA	Oat (1)
					ENB + 15ADON	Cc Maize (2)
Total		*3*		*2*		*12*
3 Mycotoxin	HT-2 + ENB + ENB1	Oat (1)	ENA1 + ENB + ENB1	Oat (1)	ENA1 + ENB + BEA	Sulla + Oat (1)
	ENA1 + ENB + ENB1	Triticale (1)				
	HT-2 + DON + ENB	Triticale (1)				
Total		*3*		*1*		*1*
4 Mycotoxin					15ADON + ENA1 + ENB + ENB1	Oat (1)
					HT-2 + ENA1 + ENB + ENB1	Oat (1)
					HT-2 + ENA1 + ENB + BEA	Oat (1)
					HT-2 + FB2 + ENA1 + ENB	Sulla + Oat (1)
Total						*4*
5 Mycotoxin					HT-2 + ENA1 + ENB + ENB1 + BEA	Oat (1)
Total						*1*

651 µg/kg (Driehuis et al., 2008); in Uruguay, in wheat silage at 6007 µg/kg and in Germany, it was higher than 300 µg/kg (Driehuis et al., 2008). However, other authors have set that the presence of DON in silage is sporadic and if any, at low concentrations (Gallo et al., 2015; Wzmbacq et al., 2016) or on its modified forms (masked) as during the fermentation process they are able to bind to microbial metabolites or their cell walls (Berthiller et al., 2013; Kovač et al., 2018). Here it was revealed that levels and contamination frequency of DON were higher on pre-fermented samples. This suggest that DON contamination decrease during storage. Ensiling process affect fungal growth as pH decrease lead to unsuitable acidified environment and oxygen is consumed reaching anaerobiosis (Manisfield et al., 2005; Boudra & Morgavi, 2008). In a recent study it was indicated that during the ensiling, the presence of lactic acid bacteria increases which contributes to detoxification effect (Ma et al., 2017). Also, del Palacio et al. (2016) have stated that a reduction of the incidence and levels of 43% wheat silage in DON contamination was detected after a 4-months storage period.

This is the first report on emerging mycotoxins performed in silage samples from Tunisia. In compliance with our results, it has been confirmed the presence of ENs and BEA in silage (Sorensen et al., 2008; Shimshoni et al, 2013; Mcelhinney et al, 2015) with sporadic occurrence and/or low frequencies (Sulyok et al, 2010; Shimshoni et al, 2013). For HT-2, which was mainly found in the region of Bizerte, is known to be produced in hot and humid climate (Fels-Klerx, 2010; Xu et al., 2014). Regarding ZEA, higher mean levels have been detected in comparison with the present study as reported by Schmidt et al. (2015) at (181 ± 278) µg/kg and Driehuis et al. (2008) at 146 µg/kg. In addition, ZEA has been frequently reported in silage fodder (Schmidt et al., 2015; Storm et al., 2014).

3.2. Occurrence of mycotoxins according the type of silage

The available data in the litterature about the incidence of mycotoxins in silage are mainly focused on maize, grass and their mixture. Very few information on other types of silage have been published. In the present study, it has been included eight different silage products, four with unique silage variety (oat, tritical, rayegrass and artichoke) and four with combination of two silage variety (oat + triticale, oat + barley, sulla + oat and sulla + rayegrass). According to the studied matrix and the different sampling stages, it was observed that triticale and oat presented the highest incidence of mycotoxins (Table 3). Triticale had four different mycotoxins in both P1 (DON, and ENB) and P2 (ZEA and BEA); while, seven toxins were observed in oat at both P2 (DON, ENA1, ENB and ENB1) and P3 (DON, ENB1, ENB, BEA and HT-2) stages and in artichoke (ENB, BEA and TENT) in the three sampled stages.

Regarding to silage samples containing mixtures of two varieties of forage, the most contaminated was sulla + oat combination, which presented more than one mycotoxin, and oat + triticale which presented only DON at the highest value (2309 µg /kg at P1). Sulla + oat samples presented DON (906 µg/kg) at P3 and ENB in slightly decresing amounts from P1 to P3 (from 0.7 to 0.5 µg/kg). The other two mixtures presented a trace contamination in oat + barley at 0.2 µg ENB/kg as well as HT-2 present in one sample at 22.4 µg/

kg. Our results confirmed that the occurrence of mycotoxins is higher in cereals (oat and triticale) than in the other analyzed plant materials or the mixtured silage samples, since mycotoxins mainly fusariotoxins are frequently found contaminating cereals, which may represent a higher source of nutrients (Oueslati et al., 2018).

3.3. Co-occurrence of mycotoxins

In the present investigation, 11 samples were contaminated with more than one mycotoxin. Table 4 shows the combination of *Fusarium* mycotoxins co-occurring in each sample and its main composition. ENB was the major mycotoxin present in these samples. While, oat based samples were the most co-contaminated matrix with up to four mycotoxins (HT-2 + ENB1 + ENB + BEA) found at P3 in one sample. Moreover, other triticale samples at P3 were contaminated with four mycotoxins simultaneously (DON + ENA1 + ENB + EN B1): with three combined mycotoxins ENA1 + ENB + EN B1 and HT-2 + DON + ENB in triticale and oat, respectively at P3 and ENA1 + ENB + ENB1 in oat at P2. Lastly, with two combined mycotoxins DON + ENB in oat at P2 and P3; ENB + BEA in artichoke sample at P2; and DON + ENB in triticale at P1 were observed. In summary, mycotoxins combination were found mainly in oat based samples at the three stages, in seven samples and only two samples of triticale.

Overall, the diversity of the mycotoxins combination was observed mainly at P3 with four different combinations of mycotoxins. In fact, the ready-to-use silage was brought back to the aerobic conditions which is a critical point that ensures the good quality of the silage as the optimal conditions may induce the growth of the existing fungi and the mycotoxins production again. Furthermore, at this stage, farmers also manage their silage products by adding concentrate supplements with regards to the targeted performances of the livestock. This may include other sources of mycotoxins by adding cereal grains (barley, maize, wheat…), plants by-products (tomatos, beets, olives, dates…) and also oilseeds (pea, soy, lupine…). It is a common practice to meet the specific needs of each farmers that have to be investigated further to ensure a safe silage quality at every scale. Although the frequency of co-occurrence of several mycotoxin in samples analyzed are low, observing the co-occurrence of mycotoxins with different chemical properties and modes of action in silage could imply a real issue if considering additive and/or synergistic effects; especially in dairy cows, which are economically and industrially of high importance for their derived products.

4. Conclusion

In the present study, results contribute to a better knowledge of natural mycotoxins contamination of green forage intended to the silage production as no data were published previously in Tunisia.

It is revealed that mycotoxins in silage from Tunisia depends on its composition, especially if they are oat based. Its presence starts in the pre-harvest season without exceeding EU levels along the silage production. Mycotoxin incidence may decrease during the ensiling process due to the fermentation, modification or masking of mycotoxins. Although the incidence of mycotoxin is reduced, amounts increase with the storage period, which decreases the transfer of mycotoxins to the animal by-products and safety in food consumers. The obtained data did not recognize any hazard on the ruminants or further derived products for consumers health in Tunisia. Still, it is important to promote the constant silage monitoring to collect information and build data on the toxigenic fungi dynamics and their mycotoxins under different environmental conditions. Further, a continuous enhancement of the silage quality and mycotoxins management and control should be implemented by developping ensilable plant material with a good resistant to fungal attack and environmental factors.

Acknowledgement

This research has been supported by the Spanish Ministry of Economy and Competitiveness [AGL2016-77610-R]. Special thanks to all members of the Ministry of Agriculture of Tunisia for their participation in the sampling procedure. Finally, thanks to the PhD student A. Mannai and Professor H. Ben Salem for their efforts to supply the samples and their availability.

Declaration of Competing Interest

None.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2020.06.029.

References

Akande, K.E., Abubakar, M.M., Adegbola, T.A., Bogoro, S.E., 2006. Nutritional and health implications of mycotoxins in animal feeds: a review. Pakistan J. Nutrit. 5 (5), 398–403. https://doi.org/10.3923/pjn.2006.398.403.

Ben Salem, H., Khlij, E., Ben Hamouda, M., Gabiña, D., 2011. Mutations des systèmes alimentaires des ovins en Tunisie et place des ressources alternatives : CIHEAM / IRESA / OEP, 2011. 29-39. http://om.ciheam.org/article.php?IDPDF = 801445.

Berthiller, F., Crews, C., Dall'Asta, C., De Saeger, S., Haesaert, G., Karlovsky, P., Oswald, I.P., Seefelder, W., Speijers, G., Stroka, J., 2013. Masked mycotoxins: a review. Mol. Nutrit. Food Res. 57, 165–186. https://doi.org/10.1002/mnfr.201100764.

Boudra, H., Morgavi, D.P., 2008. Reduction in Fusarium toxin levels in maize silage with low dry matter and storage time. J. Agric. Food. Chem. 56, 4532–4538. https://doi.org/10.1021/jf800267k.

Boudra, H., 2009. Les mycotoxines dans les fourrages : un facteur limitant insidieusement la qualité des fourrages et les performances des ruminants. Fourrages 2009 (199), 265–280 https://abiodoc.docressources.fr/doc_num.php?explnum_id = 1288.

Cogan, T., Hawkey, R., Higgie, E., Lee, M.R.F., Mee, E., Parfitt, D., Raj, J., Roderick, S., Walker, N., Ward, P., Wilkinson, J.M., 2016. Silage and total mixed ration hygienic quality on commercial farms: implications for animal production. Grass Forage Sci. 71, 339–352. https://doi.org/10.1111/gfs.12265.

del Palacio, A., Bettucci, L., Pan, D., 2016. Fusarium and Aspergillus mycotoxins contaminating wheat silage for dairy cattle feeding in Uruguay. Brazilian J. Microbiol. 47 (4), 1000–1005. https://doi.org/10.1016/j.bjm.2016.06.004.

Driehuis, F., Spanjer, M.C., Scholten, J.M., Giffel, M.C., 2008. Occurrence of mycotoxins in feedstuffs of dairy cows and estimation of total dietary intakes. J. Dairy Sci. 91, 4261–4271. https://doi.org/10.3168/jds.

Duarte, S.C., Lino, C.M., Pena, A., 2011. Ochratoxin A in feed of food-producing animals: an undesirable mycotoxin with health and performance effects. Vet. Microbiol. 154, 1–13. https://doi.org/10.1016/j.

European Commission (EC), 2002. Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC). Off. J. Eur. Union L221, 8–23 http://data.europa.eu/eli/dec/2002/657/oj.

European Commission (EC), 2006. Commission Regulation 1881/2006 of 555 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Union L364, 5–24 http://data.europa.eu/eli/reg/2006/1881/oj.

Fels-Klerx, H.J., 2010. Occurrence data of trichothecene mycotoxins T-2 toxin and HT-2 toxin in food and feed. EFSA Supp. Publ. 7, 7. https://doi.org/10.2903/sp.efsa.2010.EN-66.

Gallo, A., Giuberti, G., Frisvad, J.C., Bertuzzi, T., Nielsen, K.F., 2015. Review on mycotoxin issues in ruminants: Occurrence in forages, effects of mycotoxin ingestion on health status and animal performance and practical strategies to counteract their negative effects. Toxins (Basel) 7, 3057–3111. https://doi.org/10.3390/toxins7083057.

Gonzales Pereyra, M.L., Alonso, V.A., Sager, R., Morlaco, M.B., Magnoli, C.E., Astoreca, A.L., Rosa, C.A.R., Chiacchiera, S.M., Dalcero, A.M., Cavaglieri, L.R., 2008. Fungal and selected mycotoxins from pre- and postfermented maize silage. J. Appl. Microbiol. 104, 1034–1041. https://doi.org/10.1111/j.1365-2672.2007.03634.x.

Jemaa, T., Huguenin, J., Moulin, C.H., Najar, T., 2016. Les systèmes d'élevage de petits ruminants en Tunisie Centrale : stratégies différenciées et adaptations aux transformations du territoire. Cahiers Agric. 25, 45005. https://doi.org/10.1051/cagri/2016030.

Juan, C., Berrada, H., Mañes, J., Oueslati, S., 2017. Multi-mycotoxin determination in barley and derived products from Tunisia and estimation of their dietary intake. Food Chem. Toxicol. 103, 148–156. https://doi.org/10.1016/j.fct.2017.02.037.

Kosicki, R., Błajet-Kosicka, A., Grajewski, J., Twarużek, M., 2016. Multiannual mycotoxin survey in feed materials and feedingstuffs. Anim. Feed Sci. Technol. 215, 165–180. https://doi.org/10.1016/j.anifeedsci.2016.03.012.

Kovač, M., Šubarić, D., Bulaić, M., Kovač, T., Šarkanj, B., 2018. Yesterday masked, today modified; what do mycotoxins bring next?. Arch. Ind. Hygiene Toxicol. 69 (3), 196–214. https://doi.org/10.2478/aiht-2018-69-3108.

Ma, Z.X., Amaro, F.X., Romero, J.J., Pereira, O.G., Jeong, K.C., Adesogan, A.T., 2017. The capacity of silage inoculant bacteria to bind aflatoxin B1 in vitro and in artificially contaminated corn silage. J. Dairy Sci. 100, 7198–7210. https://doi.org/10.3168/jds.2016-12370.

McElhinney, C., Danaher, M., Elliott, C.T., O'Kiely, P., 2015. Mycotoxins in farm silages – a 2-year Irish national survey. Grass Forage Sci. 2015. https://doi.org/10.1111/gfs.12191.

Mohamed-Brahmi, A., Khaldi, R., Khaldi, G., 2010. L'élevage ovin extensif en Tunisie Disponibilités alimentaires et innovations pour la valorisation des ressources fourragères locales. ISDA, 1–12 https://hal.archives-ouvertes.fr/hal-00521129.

Olivier, J.T., Blein, R., Touré, O., 2015. Les stocks de proximité en céréales et aliments du bétail : entre gestion des crises et régulation des marchés. P1-85. http://www.inter-reseaux.org/IMG/pdf/capi-stocks-proximite-vf.pdf.

Oueslati, S., Berrada, H., Mañes, J., Juan, C., 2018. Presence of mycotoxins in Tunisian infant foods samples and subsequent risk assessment. Food Control 84, 362–369. https://doi.org/10.1016/j.foodcont.2017.08.021.

Panasiuk, L., Jedziniak, P., Pietruszka, K., Piatkowska, M., Bocian, L., 2019. Frequency and levels of regulated and emerging mycotoxins in silage in Poland. Mycotoxin Res. 35, 17–25. https://doi.org/10.1007/s12550-018-0327-0.

Richard, E., Heutte, N., Bouchart, V., Garon, D., 2009. Evaluation of fungal contamination and mycotoxin production in maize silage. Anim. Feed Sci. Technol. 148 (2-4), 309–320. https://doi.org/10.1016/j.anifeedsci.2008.02.004.

Schmidt, P., Novinski, C.O., Junges, D., Almeida, R., de Souza, C.M., 2015. Concentration of mycotoxins and chemical composition of corn silage: a farm survey using infrared thermography. J. Dairy Sci. 98 (9), 6609–6619. https://doi.org/10.3168/jds.2014-8617.

Shimshoni, J.A., Cuneah, O., Sulyok, M., Krska, R., Galon, N., Sharir, B., Shlosberg, A., 2013. Mycotoxins in corn and wheat silage in Israel. Food Addit. Contam.: Part A 30 (9), 1614–1625. https://doi.org/10.1080/19440049.2013.802840.

Stanciu, Oana, Juan, Cristina, Miere, Doina, Loghin, Felicia, Mañes, Jordi, 2017. Occurrence and co-occurrence of *Fusarium* mycotoxins in wheat grains and wheat flour from Romania. Food Control 73, 147–155. https://doi.org/10.1016/j.foodcont.2016.07.042.

Storm, I.M.L.D., Rasmussen, R.R., Rasmussen, P.H., 2014. Occurrence of pre- and post-harvest mycotoxins and other secondary metabolites in Danish maize silage. Toxins (Basel) 6, 2256–2269. https://doi.org/10.3390/toxins6082256.

Storm, I.M., Sørensen, J.L., Rasmussen, R.R., Nielsen, K.F., Thrane, U., 2008. Mycotoxins in silage. Stewart Postharvest Rev. 4 (6), 1–12. https://doi.org/10.2212/spr.2008.6.4.

Sulyok, M., Krska, R., Schumacher, R., 2010. Application of an LC–MS/MS based multi-mycotoxin method for the semi-quantitative determination of mycotoxins occurring in different types of food infected by moulds. Food Chem. 119, 408–416. https://doi.org/10.1007/s00216-007-1542-2.

Xu, X., Madden, L.V., Edwards, S.G., 2014. Modeling the effects of environmental conditions on HT2 and T2 toxin accumulation in field oat grains. Phytopathology 104, 57–66. https://doi.org/10.1094/phyto-03-13-0070-r.

Yiannikouris, A., Jouany, J.P., 2002. Mycotoxins in feeds and their fate in animals: a review. Animal Res. 51, 81–99. https://doi.org/10.1051/animres:2002012.