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Occurrence of mycotoxins and mycotoxigenic fungi in silage from the north of Portugal at feed-out

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ABSTRACT

Maize and grass silages are important dietary components for ruminant livestock that influence the quality of animal products for human consumption, such as milk, in many parts of the world. Infection of plants by fungi able to produce mycotoxins, either in the field or post-harvest, can result in a decrease of silage nutritional quality and, consequently, in milk quality. In this study, 45 maize and grass silage samples were collected from 25 dairy farms located in the north of Portugal. The occurrence of fungi was evaluated in samples, the most frequently isolated species being *Aspergillus fumigatus*, *Dipodascus geotrichum*, *Mucor circinelloides*, *Penicillium paneum,* and *Aspergillus flavus*. The mycotoxigenic profile of the fungal species was studied using the ultra-high-performance liquid chromatography coupled to mass spectrometry–ion trap–time-of-flight (UHPLC–MS–IT–TOF) detection. In addition, a new method based on a QuEChERS extraction followed by the UHPLC- tandem mass spectrometry (UHPLC-MS/MS) detection was developed for simultaneous analysis of 39 mycotoxins in silage. A high co-occurrence of *Fusarium* mycotoxins was found, although at low levels of contamination. Deoxynivalenol and beauvericin were found in more than 82% of maize silage samples. It can be highlighted the low occurrence of *Penicillium* and *Aspergillus* toxins in the maize and grass silages studied despite the frequent detection of species of both genera.

1. Introduction

Dairy cattle feeding is the first step in the milk supply chain. In many countries, milk is produced primarily on semi-intensive and intensive farms, where high-yielding dairy cows are confined seasonally or often throughout the year, and the daily ration is based on forages produced and stored on the farm, mainly silages (FAO, 2014). In pasture-based dairy systems, silages are used when fresh pasture is not available, such as in winter and summer (Wilkinson and Davies, 2013). Silages are forage feeds that have a low pH resulting from the natural lactic fermentation of soluble carbohydrates of high moisture crops under anaerobic conditions. Maize and grass are the most important crops for silage making in Europe, North America, Australia, New Zealand and Japan (Wilkinson and Toivonen, 2003). Of particular interest is the whole-plant maize silage, which may represent 50–70% of the diet for a dairy cow consuming around 26 kg dry matter per day (Drackley et al., 2006; Driehuis et al., 2008).

Production of high-quality silages is dependent on several factors, but their correct preservation mainly depends on achieving anaerobic conditions. Ensiled materials are excellent substrates for yeast and fungal growth in case they are exposed to air. In horizontal silos and piles, spoilage can be the consequence of insufficient compression of the forage or inadequate management to exclude air from entering under the plastic (Borreani et al., 2018). Most importantly, even in good quality silages, aerobic spoilage is practically unavoidable during feed-out, when the silo face is open to remove silage for feeding (Woolford, 1990). After opening the silo face, the aerobic spoilage is promptly triggered by yeasts, which cause an increase in pH and temperature that

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favor the activity of other aerobic microorganisms that have a slower growth, such as filamentous fungi (Gallo et al., 2015).

Fungal spoilage in silages is associated with nutrient and dry matter losses, reduction in palatability, mycotoxin production, and reduced feed intake (Rodríguez-Blanco et al., 2021). Mycotoxins are low molecular weight compounds produced by fungi that elicit a toxic response in humans and vertebrate animals. Mycotoxin contamination of silage can occur first in the field, mainly with toxins produced by *Fusarium* species, and post-harvest mainly with *Penicillium* and *Aspergillus* toxins (Panasiuk et al., 2019). Several works have studied the species of fungi associated with aerobic spoilage in silages, although in a number of works only at the genus level (Cheli et al., 2013). Species of the genera *Aspergillus*, *Fusarium, Penicillium, Alternaria, Mucor, Byssochlamys,* and *Monascus* have been frequently reported, many of them able to produce mycotoxins (Cheli et al., 2013).

Although more than 300 mycotoxins have been identified, only a few have received scientific interest. The most studied are regulated toxins, namely aflatoxins (AFs), ochratoxin A (OTA), patulin (PAT), citrinin (CTN), fumonisins (FBs), zearalenone (ZEN), and trichothecenes such as deoxynivalenol (DON), T-2 toxin and HT-2 toxin. In addition to regulated mycotoxins, beauvericin (BEA), enniatins (ENNs), roquefortine C (RC), and mycophenolic acid (MPA) have been also found in silages (Alonso et al., 2013; Van Pamel et al., 2011). These compounds can be classified as emerging mycotoxins since they are neither routinely determined nor legislatively regulated; however, the evidence of their incidence is rapidly increasing (Gruber-Dorninger et al., 2017; Vaclavikova et al., 2013).

Mycotoxins in silages have been related to losses in animal performance, decreased fertility, increased disease susceptibility, and animal health problems (Cheli et al., 2013). In dairy systems, chronic exposure to mycotoxins through contaminated silages, and other ingredients of the daily ration, can be expected, leading to non-specific symptoms of disease (low resistance to infectious diseases, hormonal and metabolic imbalances, immune system impairment) (Morgavi and Riley, 2007).

Contamination of forage crops with mycotoxins seems to be unavoidable. The presence of several toxins in silage is of serious concern due to the potential additive or synergistic toxic effects in animals (Dell'Orto et al., 2015). One additional hazard for food safety is the possible carry-over of mycotoxins from feed to animal-derived products such as milk, leading to mycotoxin intake by humans. For instance, milk will be contaminated with aflatoxin M_1 (AFM_1) when cows fed a diet containing AFB_1 (Alshannaq and Yu, 2017). Due to the health hazards that suppose the presence of mycotoxins in food and feed, many countries have established maximum levels for the most toxic compounds in order to guaranty public health (Sainz et al., 2015). European Union regulations establish that feed should be safe, hygienic and should not be allowed to become moldy (EC, 2005; McElhinney et al., 2016). In this sense, maximum levels for AFB_1 have been established for feed, and there are guidance values for DON, ZEN, OTA, FBs, T-2 and HT-2 toxins (EC, 2006, 2011). Therefore, it is necessary to have methods that allow the simultaneous detection of multiple toxins, especially, regulated but also emerging and the modified forms of regulated toxins (González-Jartín et al., 2019).

Several multi-analyte methods have been developed for the analysis of mycotoxins in food, most of them based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Rodríguez et al., 2017). However, only a few methods have been optimized for the analysis of silage, especially for grass silage. This matrix contains many compounds, such as sugars, chlorophyll, and organic acids, which hinder analysis since may interact with toxin detection and cause a high matrix effect (Panasiuk et al., 2019; Rodríguez et al., 2017).

In this context, this work aimed to study the prevalence of mycotoxigenic fungal species in silages from dairy farms in the Northwest of the Iberian Peninsula, to determine the mycotoxigenic profile of the isolated fungal species, to develop a new method for the simultaneous analysis of *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* toxins in both maize and grass silage, and to investigate the presence of mycotoxins in silages.

2. Materials and methods

2.1. Chemicals and reagents

Water was purified in a Millipore Milli-Q Plus system (Millipore, Bedford, MA). Methanol, acetonitrile (ACN) acetic acid (glacial, 100%), anhydrous magnesium sulfate ($MgSO_4$), and sodium chloride (NaCl) were supplied by Panreac Quimica S.A. (Barcelona, Spain). Formic acid was purchased from Merck (Madrid, Spain), and ammonium formate from Fluka (Buchs, Switzerland). Ultrafree-MC Durapore membrane centrifugal filters (0.22 μm pore size) were from Millipore (Billerica, MA). Solid standards provided by Sigma (Madrid, Spain) were: DON, ZEN, fumonisin FB_1 (FB_1), aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), enniatin A (ENNA), enniatin A_1 ($ENNA_1$), enniatin B (ENNB), enniatin B_1 ($ENNB_1$), RC, gliotoxin (GLIO) and fusaric acid (FA). Circumdatin A was from Santa Cruz Biotechnology (Santa Cruz, CA), OTA was from Laboratorios CIFGA S.A. (Lugo, Spain), and BEA standard was from Enzo (Barcelona, Spain). Analytical standards of T-2 toxin, HT-2 toxin, neosolaniol (NEO), fumonisin B_2 (FB_2), PAT, α-zearalenol (α-ZEN), β-zearalenol (β-ZEN), 3-acetyldeoxynivalenol (3-AC-DON), 15-acetyldeoxynivalenol (15-AC-DON), deoxynivalenol-3-glucoside (DON-3-Gluc), deepoxy-deoxynivalenol (DOM-1), zearalanone (ZOL), α-zearalanol (α-ZOL), β-zearalanol (β-ZOL), CTN, hydrolyzed fumonisin B_1 (Hydro-FB_1), sterigmatocystin (STC), MPA, T-2 triol, diacetoxyscirpenol (DAS), fusarenon X (FX), alternariol (AOH), alternariol methyl ether (AME), were from Romer Labs (Tulln, Austria). Ion trap time-of-flight mass spectrometry (MS-IT-TOF) calibration solution (Reference: 641225-06613-08) was from Shimadzu (Kyoto, Japan).

2.2. Sampling

From September 2019 to September 2020, 45 silage samples (39 of whole-plant maize and 6 of grass) were collected at 25 intensive dairy farms located in Vila do Conde (North of Portugal). The maize silages were from bunker silos, while the grass silages from individual plastic-wrapped bales of Italian ryegrass (*Lolium multiflorum*). In the case of maize silage samples, 7 were taken in fall, 22 in winter, and 10 in summer, while grass silages were from spring. Data of monthly mean maximum temperature, mean minimum temperature, absolute maximum temperature, absolute minimum temperature and total rainfall during the period of the study were obtained from The Portuguese Institute for Sea and Atmosphere, I. P. (IPMA, IP).

The samples were collected manually from the maize and grass silages that were being used to make up the ration of the lactating cows. Composite samples (2 kg) were taken from the front face of the bunker silos, by removing subsamples at a depth of 10–15 cm following a zigzag path using clean plastic gloves. In plastic-wrapped bales of grass silage, composite samples (2 kg) were taken following also a zigzag path around the bale. Samples were placed in sealed plastic bags and kept at 4 °C. After obtaining subsamples for fungal analysis, samples were frozen until performing the analysis of mycotoxins.

2.3. Fungal isolation and identification

From each maize silage sample, Potato Dextrose Agar (PDA) (Sharlau, Barcelona, Spain) plates were prepared by placing ten pieces of stalks, ten pieces of leaves and ten pieces of kernels (five pieces by plate). For grass samples, plates were similarly prepared using pieces of stems and leaves. Plates were incubated at 24 °C in the dark. Distinct mycelia growing from pieces were separately transferred to new PDA plates to obtain monosporic fungal cultures. For this, 1 mL of sterile water was added and gently spread over the surface of each sub-culture of distinct

mycelium, scraping the surface with a sterile glass rod. The conidial suspension obtained was transferred to a sterile Eppendorf tube. Ten-fold serial dilutions were prepared from the suspension, diluted spore suspensions (1 mL) being plated individually in PDA 90 mm-plates. Plates were incubated at 24 °C in the dark for 24 h and observed under a compound microscope looking for the presence of well-spaced germinating conidia. With the aid of a sterile pointed scalpel, a piece of agar containing one germinating conidia was cut and transferred to PDA. Isolates were initially identified by observing macroscopic characteristics of mycelia and microscopic features (Carrillo, 2003; Leslie and Summerell, 2006; Samson et al., 2014; Visagie et al., 2014). Molecular identification of all fungal isolates was initially done by amplification, sequencing and phylogenetic analysis of the ITS region of rDNA (White et al., 1990). Amplification and sequencing of the following secondary barcodes were also needed: the translation elongation factor 1 alpha (*TEF1α*) for *Fusarium* isolates (O'Donnell et al., 1998), the beta-tubulin gene (*BenA*) for *Penicillium*, and *BenA* and the calmodulin gene (*CaM*) for *Aspergillus* species (Glass and Donaldson, 1995; Hong et al., 2005; Peterson et al., 2005; Samson et al., 2014; Visagie et al., 2014). Multiple sequence alignment-based phylogenetic approaches were applied to identify fungal species when two or more DNA barcodes were used.

2.4. LC-MS detection

In order to quantify the level of mycotoxins in silage samples, a 1290 Infinity Ultra high-performance liquid chromatography (UHPLC) system interfaced to a 6460 Triple Quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany) was employed.

A UHPLC system interfaced to an IT-TOF instrument (Shimadzu, Kyoto, Japan) was employed to analyze fungal extracts since it acquires data in a full scan mode allowing to perform a nontarget analysis.

In both cases, the separation was done using a 100 mm × 2.1 mm (inside diameter), 1.8 μm, Waters ACQUITY HSS T3 column (Waters, Milford, MA); the temperature was maintained at 40 °*C*. Mobile phases were water containing 0.1% formic acid and 5 mM ammonium formate (mobile phase A), and methanol (mobile phase B). Elution gradients are shown in Table S1; the flow rate was maintained at 0.3 mL/min, and the injection volume was set at 5 μL.

The Agilent 6460 triple quadrupole mass spectrometer was equipped with an electrospray ionization source (ESI) using Agilent Jet Stream Technology. The ion source parameters were set as follows: capillary voltage, 4000 V; nozzle voltage in positive, 1500 V; nozzle voltage in negative, 0 V; nebulizer, 45 psi; sheath gas, 12 L/min and 400 °C; nebulizer gas, 8 L/min and 350 °C. The fragmentor voltage (FV), cell accelerator voltage (CAV), collision energy (CE) and mass transitions were optimized for each metabolite using MassHunter Optimizer software (Table S2). The conditions of the detection method had been previously optimized, and the method had been validated for the analysis of milk (González-Jartín et al., 2021a, 2021b).

The UHPLC system employed for the analysis of fungal extracts was from Shimadzu and consisted of two pumps (LC-30AD), an autoinjector (SIL-10AC) with a refrigerated rack, a degasser (DGU-20A), and a column oven (CTO-10AS). The IT-TOF instrument was equipped with an electrospray ionization (ESI) interface, and the operating conditions were as follows: nebulizing gas flow, 1.5 L/min; drying gas pressure, 105 kPa; curved desolvation line, 200 °C; heat block temperature, 200 °C; and detector voltage, 1.65 kV. The ion accumulation time was set to 20 ms with an event time of 300 ms and 3 repetitions. A full scan MS method was performed in positive and negative mode with two events for the mass ranges *m/z* 50–150 and *m/z* 150–900. A standard sample from Shimadzu was employed as an external reference to calibrate the mass range before data acquisition. The exact mass of the detected compounds is shown in Table S3.

2.5. Analysis of mycotoxins from fungal isolates

The mycotoxigenic profile of fungal isolates was evaluated in PDA cultures after one week of incubation. Three agar plugs (6-mm diameter) were cut from each culture and transferred to deactivated amber glass vials (Waters, Milford, MA). Next, 500 μL of an ACN/water/acetic acid mixture [49:50:1 (*v*/v/v)] were added, and samples were stirred in a vortex mixer for 3 min. Finally, the extract was filtered through a 0.22 μm centrifugal filter (Ultrafree-MC Durapore membrane) and stored at −20 °C until analysis.

2.6. Silage extraction optimization

A method previously developed for the extraction of mycotoxins from feedstuffs was reoptimized for the analysis of silages (González-Jartín et al., 2021a, 2021b). A sample contaminated with a mixture of toxins was extracted using the published protocol but modifying some conditions. Briefly, samples were mixed with acidified water, next ACN was added, and sample partitioning was induced with anhydrous $MgSO_4$ and NaCl. Finally, an aliquot of the extract was evaporated to dryness and reconstituted with an ACN/water/acetic acid [49:50:1 (*v*/v/v)] solution, obtaining 31.25 mg of matrix per mL of extract. Firstly, the percentage of acid for the extraction process was evaluated by comparing the concentration of toxins measured after sample extraction with water acidified with acetic acid at 0.5%, 1%, 2%, and 4%, and formic acid at 2% and 4%. Next, the proportion of water, ACN and extraction salts were studied. Samples were extracted with 10 mL of H_2O + 10 mL ACN, 10 mL of H_2O + 20 mL ACN, 20 mL of H_2O + 10 mL ACN, 20 mL of H_2O + 20 mL ACN, and phase partitioning was induced with 4 g of $MgSO_4$ and 1 g of NaCl. In addition, samples were extracted 10 mL of H_2O + 20 mL ACN and 20 mL of H_2O + 20 mL ACN, using 8 g of $MgSO_4$ and 2 g of NaCl for inducing phase partitioning.

2.7. Mycotoxin extraction from silage samples

Samples were thoroughly homogenized, and a 2.5 g portion was weighed in a 50 mL Falcon tube. Then, they were extracted with 10 mL of acetic acid (1%) by shaking for 5 min using a vortex mixer. Next, 20 mL of ACN were added, and the contents were stirred in a mixer for 5 min. Thereafter, a mixture of 8 g of $MgSO_4$ and 2 g of NaCl was added and mixed for 1 min. Subsequently, samples were centrifuged at 3134 ×*g* for 10 min and the upper part of the extract was transferred to a new tube. An aliquot of 100 μL of the extract was evaporated to dryness and reconstituted with 400 μL of the sample solvent, ACN/water/acetic acid [49:50:1 (*v*/v/v)]. Aliquots were filtered through 0.22 μm using centrifugal filters before UHPLC-MS/MS analysis.

2.8. Validation of the analytical procedure

Calibration curves were constructed in solvent and silage extract at nine calibration levels ranging from 25 to 6400 μg/kg (1.5 to 192 μg/kg for AFs) in order to calculate the linearity, expressed as the correlation coefficient (R), and the matrix effect. In this sense, the slope of the curves constructed in solvent and extract were compared in order to establish the signal suppression/enhancement (SSE) factor. The lowest concentration of analyte that can be detected (LOD) and the lowest concentration of analyte that can be quantified (LOQ) were calculated according to the EU-RL guidelines by analyzing blank extracts and applying the following equations: $LOQ = 3.3 \times LOD$; and $LOD = 3.9 \times \frac{Sb}{m}$ where Sb corresponds to the standard deviation of the noises of 10 blank samples, and m is the slope of the calibration curve constructed in sample solvent (Wenzl et al., 2016).

Besides, accuracy and intra-day precision were calculated using the recovery from tree replicate samples ($n = 3$) spiked at 4.8 μg/kg of AFs and at 320 μg/kg for other compounds, except FX, CTN and DOM-1,

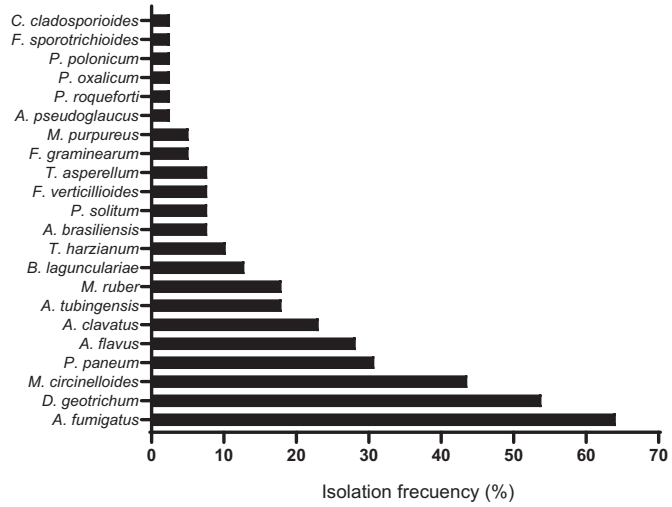

Fig. 1. Isolation frequency of fungi in maize silage. Data expressed as a percentage of positive samples.

which were spiked at 640 μg/kg. Solvent based calibration curves were employed to calculate the amount of each analyte in the extracts. In this way, apparent recoveries (R_A), relative standard deviation (RSD), and the recovery of the extraction (R_E) were evaluated. These data, together with the SSE, were calculated according to previously described equations (González-Jartín et al., 2021a, 2021b).

3. Results and discussion

The Euroregion Galicia-North of Portugal constitutes the main milk production area of the Iberian Peninsula (Chatellier and Pflimlin, 2006; Trillo-Santamaría and Paül, 2014). Therefore, it is important to establish the occurrence of mycotoxigenic fungal species and their mycotoxins in silages from this region to characterize the risk of the presence of mycotoxins in the food chain, since dairy farms use whole-plant maize and grass silages as the base for feeding animals. In this work, the presence of fungi in silages and their potential to produce mycotoxins were studied. In addition, samples were analyzed to search for the main regulated, emerging, and modified toxins.

3.1. Fungal occurrence in silage

The internal transcribed spacer (ITS) has been widely used for molecular identification of filamentous fungi (Cheli et al., 2013). However, the use of secondary DNA barcodes and multiple sequence alignment-based phylogenetic approaches are needed for the unequivocal identification of species of the main mycotoxigenic fungal genera, namely *Aspergillus*, *Penicillium* and *Fusarium* (Lücking et al., 2020). The amplification and sequencing of secondary barcodes allowed us to identify twenty-two fungal species isolated from maize silage (Fig. 1). The most frequently detected was *Aspergillus fumigatus*, present in 64% of the analyzed samples, followed by *Dipodascus geotrichum* (54%) and *Mucor circinelloides* (44%). *Penicillium paneum* and *Aspergillus flavus* were isolated from around 30% of the samples, while *Aspergillus clavatus*, *Aspergillus tubingensis* and *Monascus ruber* were present in 20% of maize samples. In contrast, in grass silage, *A. fumigatus* was found in only one sample (17%), while *Penicillium solitum* was the most frequently isolated species (50%), followed by *Byssochlamys laguncullariae* and *A. flavus*, these two last species being both present in 33% of samples (Table 1). For ensiling, forages are chopped at harvest and stored commonly in horizontal silos (bunkers and stacks) or piles, which are then sealed with weighted plastic sheets. Also, some forage crops, such as grasses, are used for making and ensiling individual round bales, which are either kept in plastic bags or, more frequently, plastic-wrapped. The low pH and anaerobiosis achieved below the plastic help prevent the growth of spoilage microorganisms such as yeasts, molds, and undesirable aerobic bacteria, thus preserving the nutritional value of ensiled forages over extended periods. The grass silos evaluated in this study came from individual plastic-wrapped bales. In this type of silo, it is easier to maintain anaerobic conditions, which may justify a lower incidence of fungi compared to maize silage, obtained from bunker silos.

The fungal species more frequently identified in this work have been previously reported in silage, although important differences were found as a function of location (Alonso et al., 2013). *Aspergillus fumigatus* has been isolated worldwide from multiple materials, and it is well adapted to silage conditions with an optimum pH of growth close to the pH reached in this fodder. The incidence of this species in silage is very variable ranging from 8 to 75% of samples (Storm et al., 2010a). The hazard associated with this fungus is not only its ability to produce mycotoxins such as GLIO, but also its capacity to cause illness such as allergic reactions, aspergilloma and invasive aspergillosis in both animals and farmers (Alonso et al., 2017). In a study on maize silos in Argentina, a high abundance of *A. fumigatus* was also found (30%), although the most abundant species was *A. flavus*, which was found in up to 53% of maize silos (González Pereyra et al., 2008). In more temperate regions like France or northern Italy a low incidence of *A. flavus* has been reported (Garon et al., 2006; Spadaro et al., 2015). In the present study, *A. flavus* was isolated in 28% of the maize silage samples and 33% of grass silage samples. The presence of this fungus is of special relevance since it can produce AFs, the only mycotoxins regulated in the EU for animal feed. Furthermore, the presence of these mycotoxins in the diet of dairy cows results in carry-over to milk. AFM_1 may occur as the main metabolite of AFB_1 in animal products such as milk, and it is regulated in many parts of the world due to its toxicity (Ferrero et al., 2019).

Penicillium roqueforti sensu stricto (*s.s.*) and *Penicillium paneum* are very closely related species, referred to as *P. roqueforti sensu lato* (*s.l.*). both well-adapted to silage conditions (low oxygen levels and high lactic acid) (Wambacq et al., 2018). These species produce mycotoxins such as

Table 1
Fungal species and mycotoxins in grass silage samples. Data expressed as μg/kg. Lower than the limit of detection (<LOD), lower than the limit of quantification (<LOQ).

Sample	Species	Mycotoxins											
		FB_1	FB_2	OTA	CTN	ENNA	$ENNA_1$	ENNB	$ENNB_1$	BEA	AME	RC	STG
1	Negative	<LOD	<LOD	<LOD	<LOD	14.28	10.44	12.22	10.02	8.87	<LOD	<LOD	<LOD
2	*Byssochlamys laguncullariae*	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	40.21	<LOD
3	*Penicillium solitum*	383.10	<LOQ	341.23	701.99	12.88	9.01	12.03	6.86	8.55	63.86	39.11	46.58
4	*Aspergillus clavatus, Penicillium solitum*	<LOD	<LOD	<LOD	<LOD	15.42	<LOD	10.46	<LOQ	<LOQ	<LOD	35.16	<LOD
5	*Byssochlamys laguncullariae, Aspergillus flavus, Penicillium solitum*	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	8.82	<LOQ	8.79	<LOD	<LOD	<LOD
6	*Epicoccum nigrum, Didymella pomorum, Aspergillus tubingensis, Aspergillus flavus, Aspergillus fumigatus, Fusarium verticillioides*	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	6.57	5.67	<LOD	<LOD	<LOD

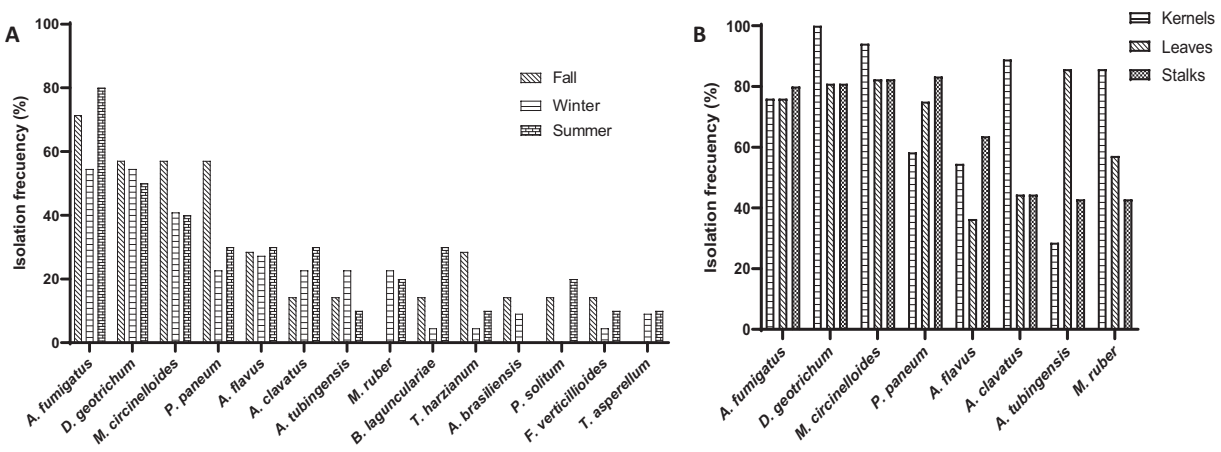

Fig. 2. Relative isolation frequency of fungi in maize silage. Percentage of contaminated samples in which the fungus was found in fall, winter, or summer (A), and in which each fungus was present in kernels, leaves or stalks (B).

roquefortines, MPA and agroclavine; in addition, *P. paneum* produces PAT. Studies carried out in Denmark, with a colder climate than in the North of Portugal, reported the presence of these two species in 96% of the silages studied (Storm et al., 2010b).

As expected, *Fusarium* species, commonly present in forage crops in the field, were found in a very low percentage of silage samples (Fig. 1). It has been previously reported their inability to persist in ensiled plant material since they do not survive at the low oxygen and low pH environment of silage (Mansfield and Kuldau, 2007). Other species isolated with a high frequency in our study were *D. geotrichum* and *M. circinelloides* although they are not related to mycotoxin production (Driehuis, 2013).

The isolation frequency was also studied in relation to the season in which samples were taken, namely fall, winter, and summer. As shown in Fig. 2A, the incidence of *A. fumigatus* varied among seasons: the lowest percentage of samples contaminated with this fungus was found in winter (54.5%), while the maximum in summer (80%). These data reinforce the hypothesis that the huge differences in the occurrence of *A. fumigatus* previously found in silage may be caused by climatic variations (Storm et al., 2010a). In fact, it was determined that the most important parameter on the growth rate of this species is temperature, with the faster growth at 37 °C (Alonso et al., 2017). This temperature corresponds to the maximum temperature (average of maximum temperatures 26.7 °C) reached in summer in the region where samples were taken (Fig. S1). On the contrary, climatic variations did not affect the incidence of *A. flavus,* although the optimal growth temperature for the fungus is 35 °C, which is usually related to the higher isolation frequency of this species in warm climates (Ferrero et al., 2019). In the case of *P. paneum* and *M. circinelloides*, the highest isolation frequency was found in fall. *Penicillium paneum* grows at an optimal temperature of 20–25 °C, but it can germinate in a broad range of both temperature and pH (Santos et al., 2020). In *in vitro* culture conditions, *M. circinelloides* shows the highest mycelium growth rate at 21 °C and a pH of 4.5 (Serna Jiménez et al., 2016). Therefore, the mild temperatures observed in fall may be the most favorable for its growth in silage. *Penicillium solitum*, a species isolated with a low frequence in samples, was detected only in fall and summer; in PDA cultures, reduced conidial germination and mycelial growth of *P. solitum* was found at temperatures lower than 20 °C, so it is likely that the low winter temperatures prevent its growth (Vico, 2010). Similarly, the occurrence of *B. laguncullariae*, detected also with low frequence, increased with higher temperatures (Fig. 2A).

In maize silos, the occurrence of fungi on leaves, stalks and kernels was studied (Fig. 2B). *Aspergillus fumigatus* was found with a high frequency in all maize plant materials. *Aspergillus clavatus*, *Monascus ruber* and, to a lesser extent, *D. geotrichum* and *Mucor circinelloides* were found mainly in kernels, *P. paneum* and *A. flavus* in stalks, and *A. tubingensis* in leaves. This is the first report on the presence of these fungal species in different plant material from maize silages at feed-out. *Aspergillus flavus* is usually found in maize grains, producing large quantities of AFs if there are favorable conditions. There are several routes of infection; it was hypothesized that one of them is *via* the stalks, since insects tunnel into this part of the plant and provide a suitable initial infection site for *A. flavus*. Next, the fungus moves through the stalk until reaching the kernel (Windham and Williams, 2007). In the present study, a similar isolation frequency was found in kernels (54%) and stalks (63%), while it was much lower in leaves (36%), which may support the previously proposed theory. *A. tubingensis* was the only fungus found with greater frequency on the leaves, this agrees with previous studies that reported this species causing disease on leaves and fruits of different plants (Khizar et al., 2020).

Table 2
Mycotoxins and other metabolites produced by the isolated fungi.

Species	Compounds
A. flavus	Aflatoxin B1, aflatoxin B2, aflavarin, aspertoxin, kojic acid
A. clavatus	Cytochalasin B/F[a], kotanin, demethylkotanin, orlandin, tryptoquivaline, 7-Hydroxy-4-methoxy-5-methylcoumarin
A. tubingensis	Asperazine, asperic acid, atromentin, aurasperone B, C, E and F, flavasperone, fonsecin, fonsecin B, fonsecinone B, funalenone, nigerazine A/B[a], nigragillin, rubrofusarin, tensidol A and B
B. laguncullariae	Byssochlamic acid, emodin, mycophenolic acid, patulin
A. brasiliensis	Aurasperone, aurasperone B/ nigerasperone B[a], carbonarone A, dehydrocarolic acid, demethylkotanin, flavasperone, fonsecin, funalenone, kotanin, nigerazine A, nigragillin, pyranonigrin A, tensidol B
P. solitum	Cyclopenin, cyclopenol, eremofortine B, penitrem A, roquefortine C, terrestric acid
F. verticillioides	Fumonisin A1, B1, B2, B3, B4, C1, C2, C3, bikaverin
A. pseudoglaucus	Auroglaucin, dihydroxyflavonone, kojic acid
D. geotrichum	ND

[a] Compounds cannot be differentiated with the employed technique.

3.2. Analysis of mycotoxins from fungal isolates

The growth of *A. fumigatus* is not limited during silage production, and, as mentioned before, this species is able to produce GLIO and other tremorgenic mycotoxins. However, the ability to produce toxins varies among strains. Therefore, to know the risk of contamination of silos, the *in vitro* production capacity of 16 *A. fumigatus* strains was established. Fungi were cultivated in PDA, a general culture medium used regularly to see the production of mycotoxins, and after 7 days of growth, the samples were analyzed by UHPLC-MS-IT-TOF, since this technology allows the tentative identification of compounds based on their exact

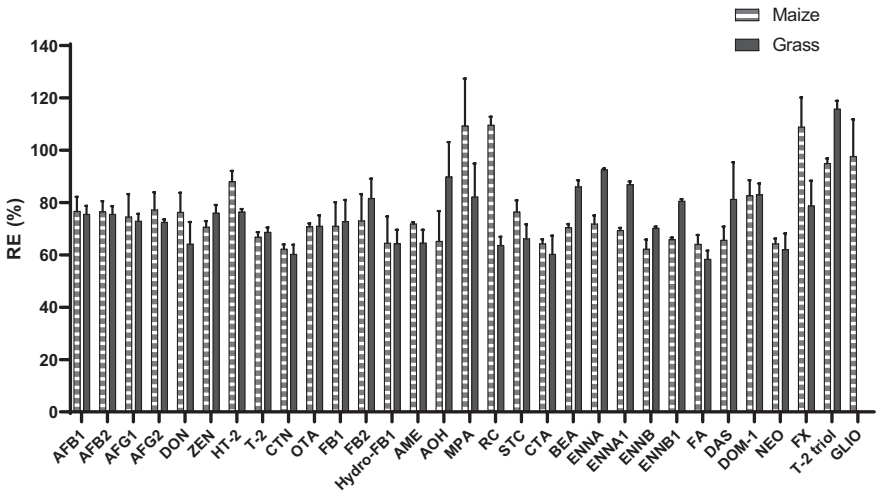

Fig. 3. Recovery of mycotoxins from maize and grass silage.

mass without the need for analytical standards (González-Jartín et al., 2018; González-Jartín et al., 2017). As shown in Fig. S2, 29 compounds were tentatively identified. However, in some cases, like sphingofungin C/sphingofungin D, compounds cannot be differentiated with the employed detection approach since they are isomers and therefore have the same mass. GLIO is considered the most toxic compound produced by this species, and it was produced by 62.5% of the analyzed strains, the fumitremorgin B by 75% and the fumigaclavine B by 95%. In Argentina, a lower number of strains able to produce these toxins was found, since only up to 48% were GLIO producers, while 21% synthetize fumitremorgin B and fumigaclavine B (Spikes et al., 2008). The higher percentage of toxin-producing strains found in the present study may be due to the more sensitive technique used LC-MS *versus* thin-layer chromatography. In addition, the production capacity of other nine species was studied. *A. flavus* is commonly isolated from multiple matrices, and approximately 50% are type B aflatoxin producers (Martins et al., 2017). As shown in Table 2, the strains tested in the present study, in addition to AFB_1 and AFB_2, produced aflavarin, aspertoxin, and kojic acid, which agree with previous studies (Uka et al., 2019). As it was expected, *B. laguncullariae* and *F. verticillioides* produced the regulated mycotoxins PAT and FBs, respectively. Some strains of *A. clavatus* are also able to produce PAT (Snini et al., 2014). This mycotoxin was not found in the isolated strain, although toxic metabolites such as cytochalasins were identified. The emerging mycotoxins RC and MPA are commonly found in silages; these compounds were identified in *P. solitum* (Fig. S5) and *B. laguncullariae* extracts, respectively. *A. tubingensis*, *A. brasiliensis*, and *A. pseudoglaucus* produced a large number of metabolites, but there are few data on their toxicity. Finally, no mycotoxins were identified in the *D. geotrichum* extract.

3.3. Silage analysis

A method previously developed for mycotoxin extraction from feedstuffs was reoptimized for the analysis of silages (González-Jartín et al., 2021a, 2021b). A contaminated maize silage sample was extracted using different conditions. First, the extraction solvent was studied (Fig. S3); the use of acetic acid led to an increase in the recovery of AFB_1, DON and FB_1. In this sense, the 1% acetic acid solution was chosen as an extraction solvent since it yielded higher recoveries for AFB_1 and FB_1. Next, the proportion of solvents and extraction salts was evaluated. The highest recoveries were obtained using 10 mL of acidified water and 20 mL of ACN (Fig. S4). Finally, the amount of dispersive salts was studied, the use of 8 g of MgSO4 and 2 g of NaCl lead to the best results (Fig. S4); therefore, this amount was selected for the analysis.

The UHPLC-MS/MS detection method has been previously validated for the analysis of regulated, emerging and modified mycotoxins in milk (González-Jartín et al., 2021a, 2021b). Therefore, the method was now in-house validated for maize and grass silage analysis in terms of sensitivity, linearity, matrix effect, recoveries, and precision for all EU-regulated mycotoxins in feed, namely AFs, DON, ZEN, FB_1, FB_2, OTA, T-2 and HT-2 toxins (EC, 2006, 2011). In addition, it was validated for the main emerging toxins produced by *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria*. Moreover, its applicability for the analysis of modified toxins was check by studying the sensitivity, linearity and matrix effect (Table S4).

LOQs were evaluated following the EU-RL guidelines (Wenzl et al., 2016). In feed, there is only a maximum limit for AFB_1. Although the legislation does not specifically contemplate silage, a maximum level of 20 μg/kg has been established for all feed materials, and, in the case of complementary and complete feed for dairy cattle, the maximum level allowed is 5 μg/kg. In the present method, the LOD for AFB_1 in both silages was lower than 2.4 μg/kg, which will allow the use of the method for the analysis of AFs with the sensitivity required by the legislation (EC, 2011; EC_401, 2006). In the case of toxins for which recommendations have been established, LOQs were up to 100 times lower than the maximum proposed values and therefore the sensitivity of the method is also sufficient to detect these mycotoxins (EC, 2006). The LOQs for emerging toxins were generally lower than 50 μg/kg, while for modified toxins vary between 37 and 294 μg/kg. Therefore, the proposed method allows the simultaneous detection of multiple mycotoxins with adequate sensitivity. However, although the initially validated method included PAT, this mycotoxin was excluded from silo analysis since the LOD was higher than 1500 μg/kg (González-Jartín et al., 2021a, 2021b). Similarly, most of the multi-detection methods validated for the analysis of mycotoxins in silos do not include PAT (Dell'Orto et al., 2015). The linearity was evaluated in matrix-matched calibration curves in a wide range of concentrations generally varying from 25 to 6400 μg/kg (1.5 to 192 μg/kg for AFs). A linear response was obtained for all mycotoxins with R values were higher than 0.995. Next, the matrix effect was calculated as the SSE by comparing the slope of the calibration curves constructed in solvent and in matrix. SSE values lower than 100% indicates matrix suppression, while values higher than 100% indicates matrix enhancement. As shown in Table S4, the matrix effect was low with signal reductions lower than 20% (SSE factor greater than 80%). The signal was only reduced by more than 50% for 3 toxins in maize silo, namely HT-2, T-2 triol and α-ZOL. Finally, the accuracy and precision of the method were assessed based on the average and the RSD of the recoveries. Blank samples were spiked at one concentration level and extracted following the optimized protocol; toxin concentration in the extract was measured using a solvent-based calibration curve. In this

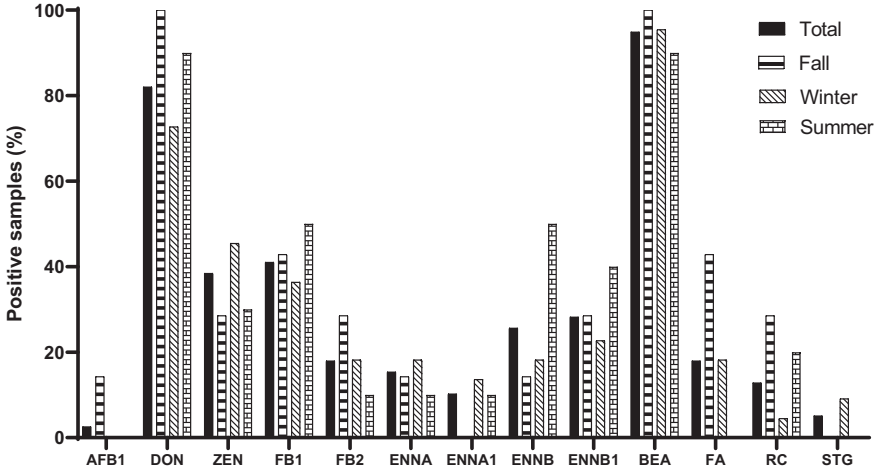

Fig. 4. Frequency of mycotoxins in maize silage.

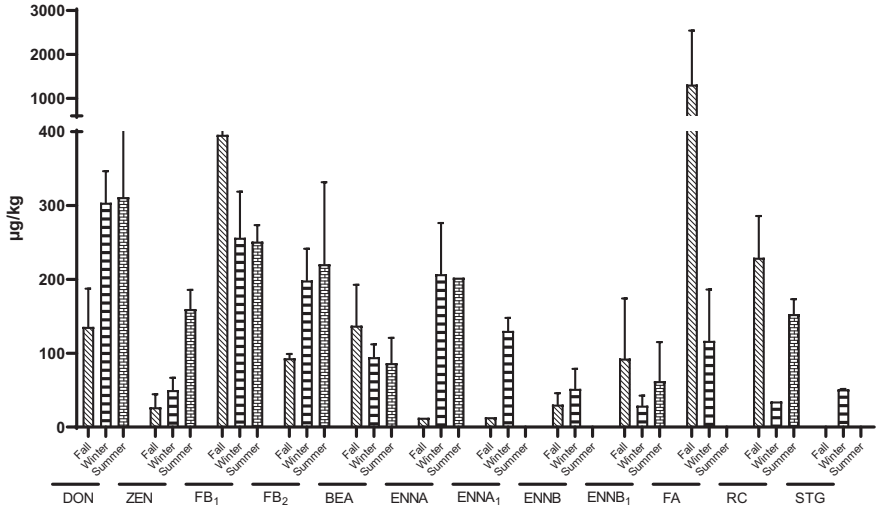

Fig. 5. Mycotoxin levels in maize silage at different sampling times.

way, the R_A was calculated and corrected with the SSE factor to obtain R_E. The intra-day precision was calculated based on the RSD of the recoveries. The mean recoveries of all compounds ranged from 58.35% to 109.64%, and the intra-day and inter-day precision from 0.42% to 18.07% (Fig. 3 and Table S4). In the EU, methods used for the analysis of mycotoxins should fulfil the performance criteria set out in the legislation, basically the recovery and the precision (EC_401, 2006). In the present method, the recovery of regulated mycotoxins varies from 64% to 88% with high precision, RSD values lower than 10%, and therefore the method conforms to European regulation.

3.4. Mycotoxin occurrence

Among the 39 mycotoxins included in the method, 13 were detected in maize silage and 12 in grass silage (Fig. 4 and Table 1). As shown in Fig. 5, none of the samples exceeded the EU maximum levels set in the EU regulations or recommendations (Table S5) (EC, 2006, 2011). The most frequently found mycotoxins were produced by *Fusarium* species. As mentioned above, species of this genus do not survive silage making; and, in fact, in the present study they have been found in a very low frequency (Fig. 1). Therefore, contamination with these toxins came from the field. In maize silo, DON was found in 82% of samples, reaching 100% during the fall (mean min. T: 11.5 °C; mean max. T: 18.9 °C; with 205,68 mm average monthly rainfall), and BEA was found in 94% of the silos, with a similar distribution among seasons (Fig. 4). Interestingly, the mean concentration of these toxins is inversely related (Fig. 5). The highest concentrations of DON were obtained in winter (mean min. T: 8.53 °C; mean max. T 16.03 °C; with 165.83 mm average monthly rainfall) and summer (mean min. T: 15.70 °C; mean max. T 26.70 °C; with 31.73 mm average monthly rainfall), with 234 µg/kg and 280 µg/kg, respectively, while those of BEA were obtained in fall, with an average of 137 µg/kg. Similar data has been previously obtained; average levels of 447 µg/kg of DON (82% of positive samples) were found in samples from Poland, although half of the positive samples contained less than 200 µg/kg (Panasiuk et al., 2019). A study that monitored the presence of mycotoxins in 10 countries from northern Europe and Turkey found 303 µg/kg of DON as average, with a 68% of positive samples (Reisinger et al., 2019). Surprisingly, a study that monitored the presence of mycotoxins in silos from the Northwest of Spain only found DON in approximately 10% of samples, with an average concentration higher than 1000 µg/kg (Dagnac et al., 2016). In the present study, the lowest average concentration of BEA was found in summer, with 86 µg/kg, and the highest in fall, with 137 µg/kg (Fig. 5). In Ireland, this emerging mycotoxin was found at 55 µg/kg, as average, in baled silages collected in winter (McElhinney et al., 2016). Lower contamination levels were found in Israel and northern Europe, with average values of 25 a 9.16 µg/kg, respectively (Reisinger et al., 2019; Shimshoni et al., 2013). The next most frequent toxins were FBs and

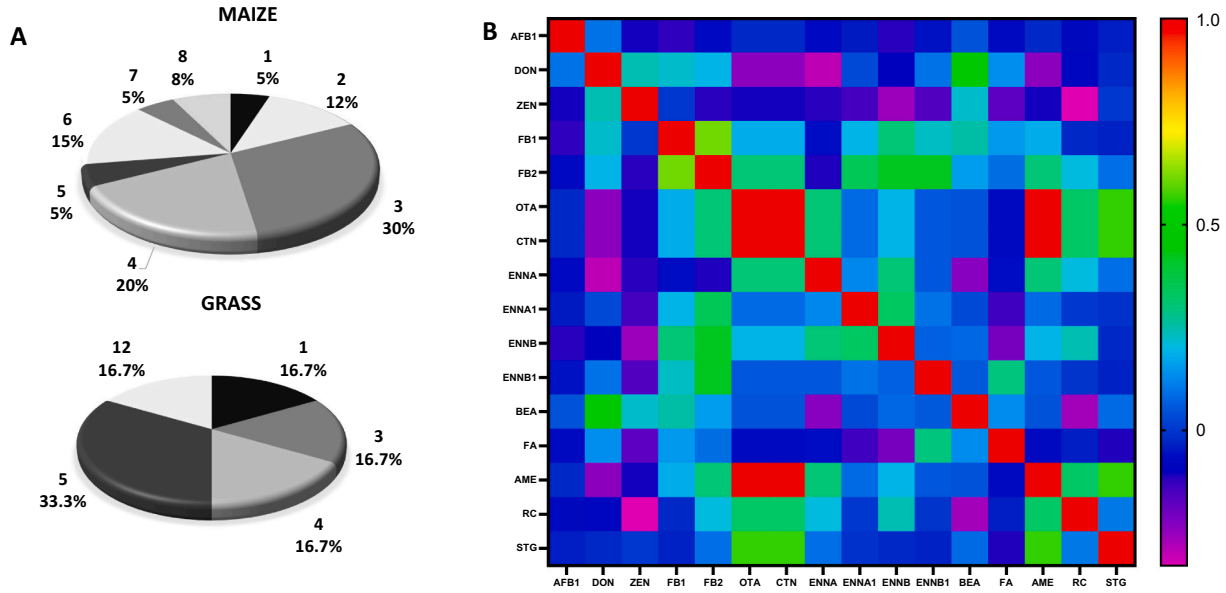

Fig. 6. Co-occurrence of mycotoxins in samples. Frequency of co-occurrence according to the number of toxins found in each sample (A), Pearson's Correlation Coefficient evaluating the co-occurrence of the toxins (B).

ZEN; FB_1 was in 41% of the samples, FB_2 in 18%, while ZEN was found in 38.5% of maize silages (Fig. 4). In the case of FBs, the occurrence can be related to the time of year; the percentage of positive samples was 36% in winter (average 239 μg/kg), 43% in fall (average 395 μg/kg), and 50% in summer (average 251 μg/kg). The higher contamination levels in fall may be the cause of the higher occurrence of FB_2 in that season, since *Fusarium* strains produce these two toxins simultaneously, but FB_1 is produced in higher amounts (Waskiewicz et al., 2010). In the case of ZEN, the average toxin concentration could be related to the time of year. In winter, silage contained 55 μg/kg as average (45.5% of positive samples), in fall 94 μg/kg (29% of positive samples) and, in summer 159 μg/kg (30% of positive samples) (Figs. 4 and 5). Although maize and maize by-products tend to have large amounts of FBs, previous studies found low occurrence in silage. In a study carried out in Northern Europe, a similar positivity of FBs was observed (35% FB_1, 29% FB_2), albeit with a lower average concentration, 60 μg/kg for FB_1 and 20 μg/kg for FB_2, respectively. In the case of ZEN, the occurrence was much higher (68%) and again with concentrations lower than 15 μg/kg (Reisinger et al., 2019). The mean concentrations of FBs found in the present study are more similar to those obtained in other studies carried out in warmer regions such as Spain (137–489 μg/kg) and Israel (303 μg/kg); the average concentration of ZEN is in the range of that obtained in samples from Spain and Netherlands (137–255 μg/kg) (Dagnac et al., 2016; Driehuis et al., 2008; Reisinger et al., 2019; Shimshoni et al., 2013). Finally, the emerging toxins of *Fusarium* FA and ENNs were found in between 10% and 28% of the samples; although the positivity of type B ENNs in silos sampled during summer reaches the 50%. In general, concentrations were lower than 80 μg/kg, although one sample contained 5000 μg/kg of FA. Similar data were found in Poland in 2005, while in Northern Europe and Turkey an average concentration of 2.5–229 μg/kg was found for these toxins during the period 2014–2018 (Panasiuk et al., 2019; Reisinger et al., 2019).

With respect to other toxins not produced by *Fusarium*, AFB_1 was detected in a maize silo sample obtained in fall (2.5% of occurrence), STG in 2 samples from winter (5.1% of occurrence), and RC in 5 samples, mainly from fall and summer (12.8% of occurrence). The concentration of STG and RC was between 34 and 285 μg/kg, while only one sample was contaminated with AFB_1 (1.4 μg/kg). The occurrence and level of contamination with these mycotoxins is in line with previous studies (Dagnac et al., 2016; Reisinger et al., 2019). The level of contamination of the grass silos was very low (Table 1); some *Fusarium* toxins such as FBs, BEA and ENNs were detected. In addition, some toxins that have not been detected in maize silage were found in this matrix, namely OTA, CTN and AME. In general, the present study reinforces that the presence of mycotoxins in grass silo is lower than in maize silo (Panasiuk et al., 2019). However, it is necessary to highlight that one of the analyzed samples contained high amounts of OTA and CTN together with other 10 mycotoxins (Fig. S6 and Table 1). The amount of OTA was around 1.5 times higher than the maximum recommended in the EU for animal feed (Table S5). A previous study showed similar maximum concentrations of OTA and CTN in non-moldy grass silages, although average concentrations were significantly lower (Tangni et al., 2013).

The RC was found in four maize samples and two grass samples although the fungi that produced this toxin were different depending on the type of silage. Grass silo samples were contaminated with *P. solitum*, while *P. paneum* was isolated from two maize silage samples and *P. roqueforti* from one. It should be mentioned that the farmers had noticed the presence of fungi in the 3 samples that have more than 100 μg/kg of RC. No RC producing strains were identified in two of the samples containing this mycotoxin, similarly, fungi producing STG, AME and AFB_1 were not found in contaminated samples. This may be due to multiple factors, such as the difficulty of having a completely homogeneous sample, or the possibility that the toxins were produced in the field and the producing strains did not survive the ensilage. Previous studies have found no correlations between specific mycotoxins and the toxin-producing fungal species (Schenck et al., 2019; Vandicke et al., 2021).

As discussed above, the fungus most frequently isolated from silos was *A. fumigatus* (Fig. 1), and 62.5% of the strains produce GLIO (Fig. S2). Therefore, it would be expected to find this mycotoxin in silage; however, it was not found. Similarly, GLIO was not detected in maize silage contaminated, among others, with *A. fumigatus* (Garon et al., 2006). *P. paneum* was isolated in 30% of silages; this strain is a producer of MPA and RC, and these toxins were found with a very low frequency. Similar observations were previously done (Storm et al., 2014).

The simultaneous occurrence of mycotoxins in the same sample was also studied (Fig. 6A). In maize silo, up to 8 mycotoxins were detected at the same time; 50% of samples contained between 3 and 4 mycotoxins, 20% between 5 and 6 and 13% more than 7 mycotoxins. In the case of

the grass silo, one sample was contaminated with 12 mycotoxins, while the rest of the samples contained between 1 and 5 toxins. Therefore, the co-occurrence of mycotoxins in silage is high; however, current regulations have not taken it into account when setting the maximum allowed or recommended levels, despite the additive or synergistic effects that may occur, increasing the total toxicity of a sample (Arroyo-Manzanares et al., 2019). Pearson's Correlation Coefficient was calculated to evaluate toxin co-occurrence (Fig. 6B and Table S6). High degree of positive correlation (coefficient value higher than 0.5) was found for DON and BEA; FB_1 and FB_2; and between OTA, CTN, AME and STG. Moderate degree of positive correlation (coefficient value between 0.3 and 0.49) was found for ENNS and FB_2; and RC with OTA and AME. It was demonstrated that some strains that produce FBs do also produce ENNs (Liuzzi et al., 2017). The correlation of ENNS with FB_2 and not with FB_1 may be due to the fact that FB_1 was found in a greater number of samples, while FB_2 was only found in samples with higher amounts of FB_1. The co-occurrence of *Fusarium* toxins found in the present study generally agree with previous findings. It should be noted that the co-occurrence of ZEN with other toxins differs between studies. Some authors found a high DON-ZEN co-occurrence, while others only notice a positive correlation between this toxin and FB_2 (Borutova et al., 2012; Kosicki et al., 2016; Panasiuk et al., 2019). In our case, a clear correlation of ZEN with other toxins was not observed.

4. Conclusion

A survey of fungi and mycotoxins in whole-plant maize and grass silages employed for feed dairy cattle in the Northwest of the Iberian Peninsula was carried out. The most frequently isolated mycotoxigenic species were *A. fumigatus*, *P. paneum*, *A. flavus*, *A. clavatus*, *A. tubingensis*, and *B. laguncularia*e. Other spoilage fungi very frequently isolated were *D. geotrichum*, *Mucor circinelloides* and *Monascus ruber*. There were important differences between the time of year and the isolation frequency of some species such as *A fumigatus*. A new method was developed and in-house validated for the analysis of mycotoxins in maize and grass silage. DON and BEA were found in the 82% and 94% of maize silage samples, respectively; high incidence of ZEN and FBs was also found. The current study shows a frequent coexistence of several mycotoxins in silages, specially several produced by *Fusarium* species, although at low levels. The low occurrence of post-harvest toxins, such as *Aspergillus* and *Penicillium* toxins, points out good silage making and storage practices in the Euroregion Galicia-North of Portugal.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2022.109556.

References

Alonso, V., Cavaglieri, L., Ramos, A.J., Torres, A., Marin, S., 2017. Modelling the effect of pH and water activity in the growth of *Aspergillus fumigatus* isolated from corn silage. J. Appl. Microbiol. 122, 1048–1056.

Alonso, V.A., Pereyra, C.M., Keller, L.A., Dalcero, A.M., Rosa, C.A., Chiacchiera, S.M., Cavaglieri, L.R., 2013. Fungi and mycotoxins in silage: an overview. J. Appl. Microbiol. 115, 637–643.

Alshannaq, A., Yu, J.-H., 2017. Occurrence, toxicity, and analysis of major mycotoxins in food. Int. J. Environ. Res. Public Health 14, 632.

Arroyo-Manzanares, N., Rodríguez-Estévez, V., Arenas-Fernández, P., García-Campaña, A.M., Gámiz-Gracia, L., 2019. Occurrence of mycotoxins in swine feeding from Spain. Toxins 11, 342.

Borreani, G., Tabacco, E., Schmidt, R.J., Holmes, B.J., Muck, R.E., 2018. Silage review: factors affecting dry matter and quality losses in silages. J. Dairy Sci. 101, 3952–3979.

Borutova, R., Aragon, Y.A., Nährer, K., Berthiller, F., 2012. Co-occurrence and statistical correlations between mycotoxins in feedstuffs collected in the Asia-Oceania in 2010. Anim. Feed Sci. Technol. 178, 190–197.

Carrillo, L., 2003. Los hongos de los alimentos y forrajes. Universidad Nacional de Salta, Argentina, pp. 91–98.

Chatellier, V., Pflimlin, A., 2006. Available from: URL. In: Dairy Systems in the European Regions of the Atlantic Area, " Green Dairy" Seminar, p. 24. https://hal.inrae.fr/hal-02814240.

Cheli, F., Campagnoli, A., Dell'Orto, V., 2013. Fungal populations and mycotoxins in silages: from occurrence to analysis. Anim. Feed Sci. Technol. 183, 1–16.

EC 401, 2006. Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off. J. Eur. Union L 70, 12–34.

Dagnac, T., Latorre, A., Fernández Lorenzo, B., Llompart, M., 2016. Validation and application of a liquid chromatography-tandem mass spectrometry based method for the assessment of the co-occurrence of mycotoxins in maize silages from dairy farms in NW Spain. Food Addit. Contam.A 33, 1850–1863.

Dell'Orto, V., Baldi, G., Cheli, F., 2015. Mycotoxins in silage: checkpoints for effective management and control. World Mycotoxin J. 8, 603–617.

Drackley, J.K., Donkin, S.S., Reynolds, C.K., 2006. Major advances in fundamental dairy cattle nutrition. J. Dairy Sci. 89, 1324–1336.

Driehuis, F., 2013. Silage and the safety and quality of dairy foods: a review. Agric. Food Sci. 22, 16–34.

Driehuis, F., Spanjer, M., Scholten, J., Te Giffel, M., 2008. Occurrence of mycotoxins in feedstuffs of dairy cows and estimation of total dietary intakes. J. Dairy Sci. 91, 4261–4271.

EC, 2005. Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. Off. J. Eur. Union L 35, 7–24.

EC, 2006. Commission recommendation (2006/576/EC) of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. Off. J. Eur. Union L 229, 7–9.

EC, 2011. Commision regulation (EU) No 574/2011 of 16 June 2011 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for nitrite, melamine, *Ambrosia* spp. and carry-over of certain coccidiostats and histomonostats and consolidating Annexes I and II thereto. Off. J. Eur. Union L 159, 7–24.

FAO, 2014. World mapping of animal feeding systems in the dairy sector. Available from: URL. FAO Rome. https://www.fao.org/publications/card/es/c/3fe753e2-9f1f-4397-acde-2bd25afb95b7/.

Ferrero, F., Prencipe, S., Spadaro, D., Gullino, M.L., Cavallarin, L., Piano, S., Tabacco, E., Borreani, G., 2019. Increase in aflatoxins due to *Aspergillus* section *Flavi* multiplication during the aerobic deterioration of corn silage treated with different bacteria inocula. J. Dairy Sci. 102, 1176–1193.

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 7, 3057–3111.

Garon, D., Richard, E., Sage, L., Bouchart, V., Pottier, D., Lebailly, P., 2006. Mycoflora and multimycotoxin detection in corn silage: experimental study. J. Agric. Food Chem. 54, 3479–3484.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 61, 1323–1330.

González Pereyra, M.L., Alonso, V.A., Sager, R., Morlaco, M.B., Magnoli, C.E., Astoreca, A.L., Rosa, C.A., Chiacchiera, S.M., Dalcero, A.M., Cavaglieri, L.R., 2008. Fungi and selected mycotoxins from pre- and postfermented corn silage. J. Appl. Microbiol. 104, 1034–1041.

González-Jartín, J.M., Alfonso, A., Sainz, M.J., Vieytes, M.R., Botana, L.M., 2017. UPLC–MS–IT–TOF identification of circumdatins produced by *Aspergillus ochraceus*. J. Agric. Food Chem. 65, 4843–4852.

González-Jartín, J.M., Alfonso, A., Sainz, M.J., Vieytes, M.R., Botana, L.M., 2018. Detection of new emerging type-A trichothecenes by untargeted mass spectrometry. Talanta 178, 37–42.

González-Jartín, J.M., Alfonso, A., Rodríguez, I., Sainz, M.J., Vieytes, M.R., Botana, L.M., 2019. A QuEChERS based extraction procedure coupled to UPLC-MS/MS detection for mycotoxins analysis in beer. Food Chem. 275, 703–710.

González-Jartín, J.M., Alfonso, A., Sainz, M.J., Vieytes, M.R., Botana, L.M., 2021. Multi-detection method for mycotoxins with a modified QuEChERS extraction in feed and

development of a simple detoxification procedure. Anim. Feed Sci. Technol. 272, 114745.
González-Jartín, J.M., Rodríguez-Cañás, I., Alfonso, A., Sainz, M.J., Vieytes, M.R., Gomes, A., Ramos, I., Botana, L.M., 2021. Multianalyte method for the determination of regulated, emerging and modified mycotoxins in milk: QuEChERS extraction followed by UHPLC–MS/MS analysis. Food Chem. 356, 129647.
Gruber-Dorninger, C., Novak, B., Nagl, V., Berthiller, F., 2017. Emerging mycotoxins: beyond traditionally determined food contaminants. J. Agric. Food Chem. 65, 7052–7070.
Hong, S.B., Go, S.J., Shin, H.D., Frisvad, J.C., Samson, R.A., 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. Mycologia 97, 1316–1329.
Khizar, M., Haroon, U., Ali, M., Arif, S., Shah, I.H., Chaudhary, H.J., Munis, M.F.H., 2020. *Aspergillus tubingensis* causes leaf spot of cotton (*Gossypium hirsutum L.*) in Pakistan. Phyton 89, 103.
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.
Leslie, J.F., Summerell, B.A., 2006. The *Fusarium* Laboratory Manual. Wiley.
Liuzzi, V.C., Mirabelli, V., Cimmarusti, M.T., Haidukowski, M., Leslie, J.F., Logrieco, A. F., Caliandro, R., Fanelli, F., Mulè, G., 2017. Enniatin and beauvericin biosynthesis in *Fusarium* species: production profiles and structural determinant prediction. Toxins 9, 45.
Lücking, R., Aime, M.C., Robbertse, B., Miller, A.N., Ariyawansa, H.A., Aoki, T., Cardinali, G., Crous, P.W., Druzhinina, I.S., Geiser, D.M., Hawksworth, D.L., Hyde, K. D., Irinyi, L., Jeewon, R., Johnston, P.R., Kirk, P.M., Malosso, E., May, T.W., Meyer, W., Öpik, M., Robert, V., Stadler, M., Thines, M., Vu, D., Yurkov, A.M., Zhang, N., Schoch, C.L., 2020. Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding? IMA Fungus 11, 14.
Mansfield, M., Kuldau, G., 2007. Microbiological and molecular determination of mycobiota in fresh and ensiled maize silage. Mycologia 99, 269–278.
Martins, L.M., Sant'Ana, A.S., Fungaro, M.H.P., Silva, J.J., Nascimento, M.d.S.d., Frisvad, J.C., Taniwaki, M.H., 2017. The biodiversity of *Aspergillus* section *Flavi* and aflatoxins in the Brazilian peanut production chain. Food Res. Int. 94, 101–107.
McElhinney, C., Danaher, M., Elliott, C.T., O'Kiely, P., 2016. Mycotoxins in farm silages – a 2-year Irish national survey. Grass Forage Sci. 71, 339–352.
Morgavi, D.P., Riley, R.T., 2007. An historical overview of field disease outbreaks known or suspected to be caused by consumption of feeds contaminated with *Fusarium* toxins. Anim. Feed Sci. Technol. 137, 201–212.
O'Donnell, K., Kistler, H.C., Cigelnik, E., Ploetz, R.C., 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proc. Natl. Acad. Sci. 95, 2044.
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.
Peterson, S.W., Vega, F.E., Posada, F., Nagai, C., 2005. *Penicillium coffeae*, a new endophytic species isolated from a coffee plant and its phylogenetic relationship to *P. fellutanum, P. thiersii* and *P. brocae* based on parsimony analysis of multilocus DNA sequences. Mycologia 97, 659–666.
Reisinger, N., Schürer-Waldheim, S., Mayer, E., Debevere, S., Antonissen, G., Sulyok, M., Nagl, V., 2019. Mycotoxin occurrence in maize silage—a neglected risk for bovine gut health? Toxins 11, 577.
Rodríguez, I., González, J.M., Botana, A.M., Sainz, M.J., Vieytes, M.R., Alfonso, A., Botana, L.M., 2017. Analysis of natural toxins by liquid chromatography. In: Fanali, S., Haddad, P.R., Poole, C.F., Riekkola, M.-L. (Eds.), Liquid Chromatography, Second edition. Elsevier, pp. 479–514.
Rodríguez-Blanco, M., Ramos, A.J., Sanchis, V., Marín, S., 2021. Mycotoxins occurrence and fungal populations in different types of silages for dairy cows in Spain. Fungal Biol 125, 103–114.
Sainz, M.J., Alfonso, A., Botana, L.M., 2015. Considerations about international mycotoxin legislation, food security, and climate change. In: Climate Change And Mycotoxins. De Gruyter, pp. 153–179.
Samson, R.A., Visagie, C.M., Houbraken, J., Hong, S.B., Hubka, V., Klaassen, C.H.W., Perrone, G., Seifert, K.A., Susca, A., Tanney, J.B., Varga, J., Kocsubé, S., Szigeti, G., Yaguchi, T., Frisvad, J.C., 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. Stud. Mycol. 78, 141–173.
Santos, J.L., Chaves, R.D., Sant'Ana, A.S., 2020. Modeling the impact of water activity, pH, and calcium propionate on the germination of single spores of *Penicillium paneum*. LWT-Food Sci. Technol. 133, 110012.
Schenck, J., Müller, C., Djurle, A., Jensen, D.F., O'Brien, M., Johansen, A., Rasmussen, P. H., Spörndly, R., 2019. Occurrence of filamentous fungi and mycotoxins in wrapped forages in Sweden and Norway and their relation to chemical composition and management. Grass Forage Sci. 74, 613–625.
Serna Jiménez, J., Quintanilla Carvajal, M.X., Rodriguez, J., Uribe, M., Klotz Ceberio, B., 2016. Development of a combined temperature and pH model and the use of bioprotectants to control of *Mucor circinelloides*. Am. J. Food Technol. 11, 21–28.
Shimshoni, J., Cuneah, O., Sulyok, M., Krska, R., Galon, N., Sharir, B., Shlosberg, A., 2013. Mycotoxins in corn and wheat silage in Israel. Food Addit. Contam.A 30, 1614–1625.
Snini, S.P., Tadrist, S., Laffitte, J., Jamin, E.L., Oswald, I.P., Puel, O., 2014. The gene *PatG* involved in the biosynthesis pathway of patulin, a food-borne mycotoxin, encodes a 6-methylsalicylic acid decarboxylase. Int. J. Food Microbiol. 171, 77–83.
Spadaro, D., Bustos-Lopez, M.d.P., Gullino, M.L., Piano, S., Tabacco, E., Borreani, G., 2015. Evolution of fungal populations in corn silage conserved under polyethylene or biodegradable films. J. Appl. Microbiol. 119, 510–520.
Spikes, S., Xu, R., Nguyen, C.K., Chamilos, G., Kontoyiannis, D.P., Jacobson, R.H., Ejzykowicz, D.E., Chiang, L.Y., Filler, S.G., May, G.S., 2008. Gliotoxin production in *Aspergillus fumigatus* contributes to host-specific differences in virulence. J. Infect. Dis. 197, 479–486.
Storm, I.M., Kristensen, N.B., Raun, B.M., Smedsgaard, J., Thrane, U., 2010a. Dynamics in the microbiology of maize silage during whole-season storage. J. Appl. Microbiol. 109, 1017–1026.
Storm, I.M., Rasmussen, R.R., Rasmussen, P.H., 2014. Occurrence of pre- and post-harvest mycotoxins and other secondary metabolites in Danish maize silage. Toxins 6, 2256–2269.
Storm, I.M.L.D., Kristensen, N., Raun, B., Smedsgaard, J., Thrane, U., 2010b. Dynamics in the microbiology of maize silage during whole-season storage. J. Appl. Microbiol. 109, 1017–1026.
Tangni, E.K., Pussemier, L., Bastiaanse, H., Haesaert, G., Foucart, G., Van Hove, F., 2013. Presence of mycophenolic acid, roquefortine C, citrinin and ochratoxin A in maize and grass silages supplied to dairy cattle in Belgium. J. Anim. Sci.Adv. 3, 598–612.
Trillo-Santamaría, J.-M., Paül, V., 2014. The oldest boundary in Europe? A critical approach to the Spanish-Portuguese border: the Raia between Galicia and Portugal. Geopolitics 19, 161–181.
Uka, V., Moore, G.G., Arroyo-Manzanares, N., Nebija, D., De Saeger, S., Diana Di Mavungu, J., 2019. Secondary metabolite dereplication and phylogenetic analysis identify various emerging mycotoxins and reveal the high intra-species diversity in *Aspergillus flavus*. Front. Microbiol. 10, 667.
Vaclavikova, M., Malachova, A., Veprikova, Z., Dzuman, Z., Zachariasova, M., Hajslova, J., 2013. 'Emerging' mycotoxins in cereals processing chains: changes of enniatins during beer and bread making. Food Chem. 136, 750–757.
Van Pamel, E., Verbeken, A., Vlaemynck, G., De Boever, J., Daeseleire, E., 2011. Ultrahigh-performance liquid chromatographic-tandem mass spectrometric multimycotoxin method for quantitating 26 mycotoxins in maize silage. J. Agric. Food Chem. 59, 9747–9755.
Vandicke, J., De Visschere, K., Ameye, M., Croubels, S., De Saeger, S., Audenaert, K., Haesaert, G., 2021. Multi-mycotoxin contamination of maize silages in Flanders, Belgium: monitoring mycotoxin levels from seed to feed. Toxins 13.
Vico, I., 2010. Temperature suppresses decay on apple fruit by affecting *Penicillium solitum* conidial germination, mycelial growth and polygalacturonase activity. Plant Pathol. J. 9, 144–148.
Visagie, C.M., Houbraken, J., Frisvad, J.C., Hong, S.B., Klaassen, C.H.W., Perrone, G., Seifert, K.A., Varga, J., Yaguchi, T., Samson, R.A., 2014. Identification and nomenclature of the genus *Penicillium*. Stud. Mycol. 78, 343–371.
Wambacq, E., Audenaert, K., Höfte, M., De Saeger, S., Haesaert, G., 2018. *Bacillus velezensis* as antagonist towards *Penicillium roqueforti* s.l. in silage: *in vitro* and *in vivo* evaluation. J. Appl. Microbiol. 125, 986–996.
Waskiewicz, A., Golinski, P., Karolewski, Z., Irzykowska, L., Bocianowski, J., Kostecki, M., Weber, Z., 2010. Formation of fumonisins and other secondary metabolites by *Fusarium oxysporum* and *F. proliferatum*: a comparative study. Food Addit. Contam.A 27, 608–615.
Wenzl, T., Haedrich, J., Schaechtele, A., Piotr, R., Stroka, J., Eppe, G., Scholl, G., 2016. Guidance Document on the Estimation of LOD And LOQ for Measurements in the Field of Contaminants in Feed and Food. Available from: URL. Publications Office of the European Union, Luxemburg. https://op.europa.eu/es/publication-detail/-/publication/200cf09a-9ad1-11e6-868c-01aa75ed71a1.
White, T.J., Bruns, T.D., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal genes for phylogenetics. In: Innis, M.A., Gelfrand, D.H., Sninsky, J. J., White, T.J. (Eds.), PCR protocols. Academic Press, San Diego, California, pp. 315–322.
Wilkinson, J.M., Davies, D.R., 2013. The aerobic stability of silage: key findings and recent developments. Grass Forage Sci. 68, 1–19.
Wilkinson, J.M., Toivonen, M.I., 2003. World Silage: A Survey of Forage Conservation Around the World. Chalcombe Publications, Lincoln.
Windham, G.L., Williams, W.P., 2007. Systemic infection of stalks and ears of corn hybrids by *Aspergillus parasiticus*. Mycopathologia 164, 249–254.
Woolford, M.K., 1990. The detrimental effects of air on silage. J. Appl. Bacteriol. 68, 101–116.