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Mycotoxin occurrence on baled and pit silages collected in Co. Meath

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Abstract

Recent studies of baled silages produced in Ireland have identified considerable filamentous fungal contamination. Many of these fungi are toxigenic, capable of producing secondary metabolites, namely mycotoxins. Mycotoxins are potentially detrimental to livestock health and some can pose a risk to consumers of animal products. Baled (n=20) and pit (n=18) silages from a sample of farms (n=38) in Co. Meath were examined to assess the occurrence of mycotoxins and ascertain whether sampling position within the pit silos (feed face vs. 3 m behind the feed face) has an effect on mycotoxin content or other chemical compositional variables. Of the 20 mycotoxins assayed, baled silages contained [mean of positive values (no. of values in mean)] mycotoxin concentrations (µg/kg dry matter) of beauvericin 36 (2), enniatin (enn.) A 9.3 (3), enn. A_1 54 (8), enn. B 351 (9), enn. B_1 136 (10), mycophenolic acid (MPA) 11,157 (8) and roquefortine C (Roq. C) 1037 (8) and pit silages contained beauvericin 25 (2) enn. A_1 18 (2), enn. B 194 (9), enn. B_1 57 (3), MPA 287 (6), Roq. C 3649 (6) and zearalenone 76 (1). There was no difference (P>0.05) observed in the mycotoxin concentrations between baled and pit silages, and 11 of the 20 mycotoxins assayed were below the limits of detection. The position of sampling had no effect on the mycotoxin concentration detected in pit silages. It is concluded that mycotoxin concentrations detected in these pit and baled silages in Co. Meath did not exceed EU regulation or guidance limits, and that similar chemical composition and mycotoxin concentration values occurred at the pit silage feed face and 3 m behind this feed face.

Keywords

Silage • mycotoxins • fungi • baled • pit

Introduction

Approximately 1.05 million hectares of land are used annually for the production of silage (CSO, 2011), one-quarter of the total area farmed in Ireland. After grazed grass, grass silage is the most important crop produced on Irish farms, with 86% of farms producing silage (McEniry *et al.,* 2006). It is harvested using either precision chop silage (stored in horizontal pit silos, and accounting for two-thirds of the national silage area) or baled silage (wrapped in polyethylene stretch-film, and accounting for one-third of the national silage area) systems (McEniry *et al.,* 2006). The preservation of these silages depends on achieving strictly anaerobic conditions within the silo or bale to inhibit the aerobic activity of fungi and bacteria. However, a previous survey of baled silage in Ireland identified that 91% of the bales contained visible fungal mould growth (O'Brien, *et al.,* 2005), including *Fusarium* and *Penicillium,* indicating that adequately anaerobic conditions did not prevail. These moulds are toxigenic and can produce secondary fungal metabolites, namely mycotoxins.

Mycotoxins can induce a range of detrimental ailments in livestock including abortions, vomiting, lameness, immunosuppression, reduced feed intake and feed refusal (Binder *et al.,* 2007). Some mycotoxins (ochratoxin and aflatoxin B_1) detected in silage can also can be transferred through the food chain into meat and milk, respectively [European Food Safety Authority (EFSA), 2004 b; 2006). Monitoring feed for mycotoxins is requested by the EFSA and the current study includes all mycotoxins that are regulated in Commission Directive (EC) No. 32/2002 (EU Commission, 2002) and Recommendation (EC) No. 576/2006 (EU Commission, 2006).

The objectives of this study were twofold: (1) to identify the mycotoxin occurrence in baled and pit silages on farms in a mixed farming area in Co. Meath, and (2) to determine if the position of sampling within pit silos had an effect on silage mycotoxin content or on other chemical compositional characteristics. This study was a precursor

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to a national survey, that would also identify expected likely mycotoxins and their concentrations within silages.

Materials and methods

In February 2012, 38 farms were visited within a 10 km radius of Grange, Co. Meath (latitude 53.51 N, longitude 6.65 W). On 20 farms, round baled silage (nominal diameter 1.2 m and width 1.2 m) was used, and on 18 farms, precision-chop silage was stored in horizontal pit silos. A detailed questionnaire was completed for each silage collected, with information being acquired on crop type, harvesting and silage management practices.

Silage core sampling

One bale of silage was selected (the next bale ready for feeding to livestock) on each of the 20 farms. Twenty core samples (Fig. 1) were collected through the curved barrel of each bale using a motorised coring system (corer 600 mm long and 36 mm internal diameter). Cores (n=5) were collected equidistantly along each of four lines, at clock positions 0300, 0600, 0900 and 1200h, as illustrated in Fig. 1. Core samples were thoroughly mixed to produce one representative composite sample per bale. For silages stored in horizontal pit silos (n=18), horizontal core samples (n=3; 0.6 m deep) were collected from the next area ready for feeding (1–3 in Fig. 2) at the silage face, and full depth vertical core samples (n=2) were collected from 3 m behind the face (4–5 in Fig. 2). Core samples from the feed face or 3 m behind the feed face were composited to produce one representative composite sample per position in each silo.

Conventional chemical analysis

Once collected, silage samples were stored at −20ºC until required for analysis. Dry matter (DM) was determined by drying samples at 85ºC (16 h) in a calibrated oven (AEW 003549) with forced air circulation**.** Correction of silage DM for loss of volatiles during oven drying was carried out using the equation of Porter and Murray (2001). For conventional chemical and mycotoxin analyses, silage samples were dried (40ºC for 48 h) and milled (Retsch SM100) (1 mm aperture sieve). They were then assayed for *in vitro* DM digestibility (DMD) as described by Tilley and Terry (1963), with the modification that the final residue was isolated by filtration rather than centrifugation. Ash concentration was determined by complete combustion in a muffle furnace at 550ºC for 5 h. Nitrogen (N) was measured with a LECO FP-528N analyser

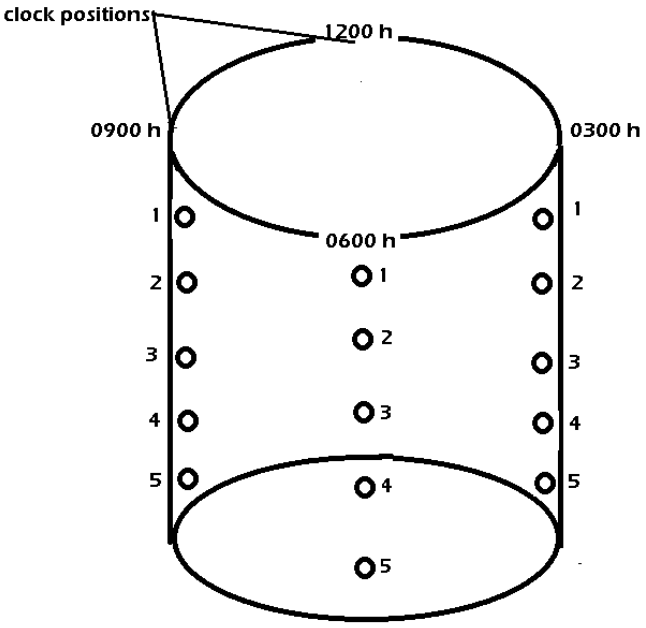

Fig. 1: Round silage bale with the coring positions (n=20) of a typical sampling plan indicated.

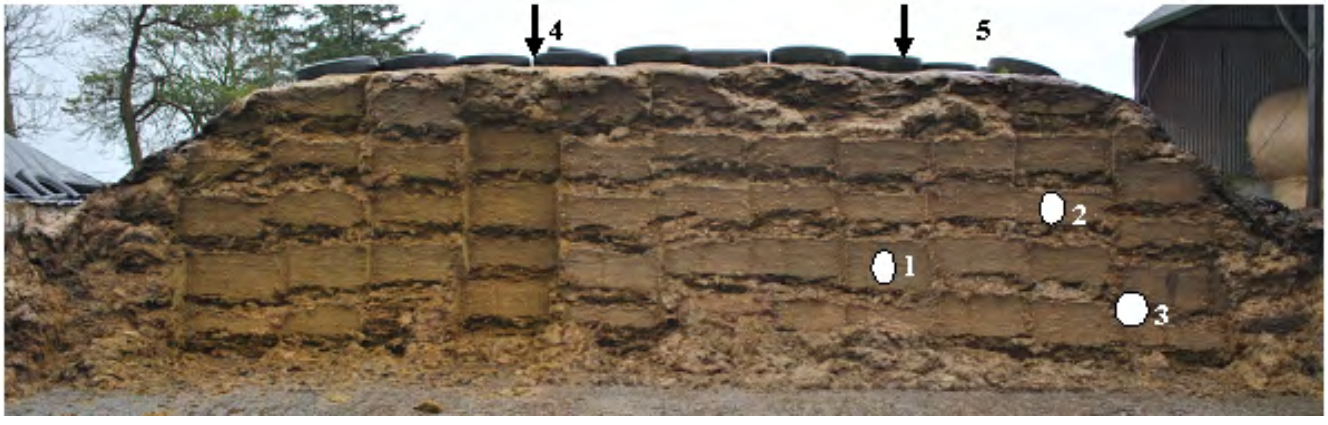

Fig. 2: Horizontal pit farm silo with the coring positions of a typical sampling plan indicated. Positions 1–3 indicate horizontal core sampling locations at the silage feed face and 4–5 indicate vertical sampling locations 3 m behind the feed face.

(Leco Corporation, St. Joseph, MI) using 990-03 method of the Association of Analytical Chemists (AOAC; 1990) and crude protein was determined as N × 6.25. The water soluble carbohydrate (WSC) concentration was determined by the automated anthrone method as described by Thomas (1977). The aqueous extract was assayed for pH (Hanna H1 8424), for l-lactic acid (catalogue number 101309084035; Boehringer, Darmstadt, Germany), for d-lactic acid (catalogue number 61306 Sigma-Aldrich, Arklow, Ireland), and for ammonia-N using the Thermo Electron Kinetic method for plasma ammonia (catalogue number TR60101, Victoria, Australia). Both lactic acid and ammonia-N were measured with an Olympus AU400 Chemistry Analyser (Shizuoka, Japan). Aqueous extract was also assayed for volatile fatty acids (acetic, propionic and butyric acids) and ethanol by gas chromatography.

Mycotoxin analysis

Liquid chromatography tandem mass spectrometry (LCMS/MS) analysis was accomplished using an Agilent 1290 liquid chromatography system coupled to Agilent 6460 triple quadrapole mass spectrometer equipped with an electrospray ionisation (ESI) source. This 16 minute analytical run can detect 20 mycotoxins (deoxynivalenol (DON), aflatoxins B_1, B_2, G_1 and G_2 (AF's), HT-2 toxin, fumonisin B_1 and B_2, mycophenolic acid (MPA), roquefortines C and E, ochratoxin A (OTA), T-2 toxin, zearalenone (ZEA), andrastin A, enniatins A_1, A, B_1 and B and beauvericin, includes all the EU regulated mycotoxins concerned with feed and has been fully validated (McElhinney *et al*., 2013). Mycotoxin extraction was carried out using a modified quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction protocol, which employed 0.1 M HCl as an extraction solvent. Prior to all LCMS/MS analysis, the supernatant is passed through a 0.2 µm Millipore syringe filter into LCMS/MS vials. The analytical column was an Acquity HSS T3 C18 (100 mm × 2.1 mm, 1.8 µm particle sizes) (Waters, Milan, Italy) preceded by a pre-filter. The flow rate of the mobile phase was 600 µl/min and the injection volume was 1 µl. The column effluent was transferred directly into the ESI source that operated in both positive and negative ion mode, with the following settings: gas temperature 200ºC; sheath gas temperature 400ºC; gas flow 8 ml/min (310 kPa) and nozzle voltage 500 v. The column oven was set to 60ºC. Eluent A was water and eluent B was methanol, both containing 2 ml acetic acid/l and 5 mM ammonium acetate. A gradient elution was performed as follows. The proportion of eluent B was kept constant for the first minute at 0%, then linearly increased from 10 to 100% from 1 to 10 min, then kept constant for 2 min before being linearly decreased back to 0% B over 2 min.

For the above analyses, methanol (MeOH, LCMS grade), methanol-d (MeOHd), acetonitrile (MeCN, LC-MS grade), ammonium acetate and magnesium sulphate ($MgSO_4$) were supplied by Sigma-Aldrich®, sodium chloride (NaCl) was purchased from Applichem, and hydrochloric acid (HCl, analaR grade) was supplied by BDH VWR International. Glacial acetic acid (17.4 M) was sourced from Merck Chemicals (Darmstadt, Germany). Water was of LCMS grade (generated by a Milli-Q gradient purification system, Millipore, Bedford, MA).

Mycotoxin standards were from Enzo Life Sciences (andrastin A, aflatoxin B_1, B_2, G_1, G_2; beauvericin, enniatins A, A_1, B, B_1; fumonisin b1, b2; HT-2, MPA, OTA, Roq. C, T-2 and Zea) and Sigma-Aldrich (DON and Zea). C-labelled MPA standard was obtained from Toronto Research Chemicals. Acquity UPLC HSS T3 C18 analytical columns were from Waters (Milan, Italy). PTFE 0.2 µm syringe filters were obtained from Merck-Millipore (Darmstadt, Germany).

Statistical analysis

Data for the comparison of conventional chemical characteristics of baled and pit silages (Table 1) were analysed by one-way analysis of variance, accounting for silage type. Values for pit silages (Table 1 and 2) are the average of the values collected at both sampling points (silage face and 3 m behind the silage face). Mycotoxin values (Table 2) were analysed by Mann–Whitney U test accounting for silage type. For comparing the effects of sampling position within pit silages, conventional chemical characteristic data (Table 3) were analysed using paired samples *t*-test, accounting for sampling position within the pit silage. Mycotoxin data (Table 4) (not normally distributed) collected from the pit silages were analysed using the Wilcoxon signed-ranked test accounting for sampling position. Mycotoxin summary statistics shown by a box plot graph (Fig. 3) include positive samples only. The R^2 values (Fig. 4) were derived using the least squares method. Mycotoxin data and other chemical characteristics were correlated using Pearson's bivariate correlation.

Results

Silage history management practices

All of the bales sampled (n=20) were made during the summer of 2011, with the highest proportion of bales being made in June (0.3) and July (0.3), followed by August (0.2) and September (0.2). Perennial ryegrass (0.8) was the dominant grass within the swards harvested. Most herbage for bales (0.9) experienced a wilting period of 2 days although some (0.1) had a wilting period greater than 2 days, and all were made without additive application. All bales were tied with netting and nominally wrapped in 4 layers of either

Table 1: Mean (s.d.) conventional chemical composition characteristics of silages collected from farms in Co. Meath in February 2012.

Variable	Silage Type					
	Baled	**s.d.**	**Pit**	**s.d.**	**s.e.d**	**Significance**
Dry matter corrected (g/kg)	373	135.0	249	38.3	33	**
Dry matter digestibility (g/kg)	572	109.4	703	47.3	27.9	***
Ash (g/kg DM)	83	27.2	83	27.3	8.8	
Crude protein (g/kg DM)	117	24.1	139	30.3	8.8	*
pH	4.7	0.55	3.9	0.41	0.16	***
Lactic acid (g/kg DM)	20	17.1	101	39.4	9.7	***
Acetic acid (g/kg DM)	6.7	6.33	27	7.4	2.2	***
Propionic acid (g/kg DM)	0.8	0.74	2.7	2.61	0.6	*
Butyric acid (g/kg DM)	6.9	7.65	4.2	5.61	2.2	
Volatile fatty acids (g/kg DM)	14	13.7	34	13.5	4.4	***
Ethanol (g/kg DM)	8.8	4.16	16	7.9	2.1	**
Fermentation products[1] (g/kg DM)	43	27.7	152	39.9	11	***
Lactic acid/fermentation products (g/g)	0.41	0.180	0.64	0.162	0.05	***
Water soluble carbohydrates (g/kg DM)	70	49.7	22	25.2	13	**
NH_3-N (g/kg N)	84	1.2	99	53.3	16.7	***

[1] Fermentation products = Lactic acid + acetic acid + propionic acid + butyric acid + ethanol.
s.d.: Standard deviation

Table 2: Mean (s.d.) mycotoxin characteristics of silages collected from farms in Co. Meath in February 2012.

Variable	Silage Type					
	Baled	**s.d.**	**Pit**	**s.d.**	**s.e.d**	**Significance**
Andrastin A (µg/kg DM)	< LOQ [1]	-	490 (n=1)	377	-	-
Beauvericin (µg/kg DM)	36 (n=2)	25.9	25 (n=2)	9.8	17.1	NS
Enniatin A (µg/kg DM)	9.3 (n=3)	6.91	< LOQ [1]	-	-	-
Enniatin A1 (µg/kg DM)	54 (n=8)	75.8	18 (n=2)	7.5	56	NS
Enniatin B (µg/kg DM)	351 (n=9)	415	194 (n=9)	100	161	NS
Enniatin B1 (µg/kg DM)	136 (n=10)	228	57 (n=3)	33.8	167	NS
Mycophenolic acid (µg/kg DM)	11157 (n=8)	25276	287 (n=6)	282	12950	NS
Roquefortine C (µg/kg DM)	1037 (n=8)	1498	364 (n=6)	224	771	NS
Zearalenone (µg/kg DM)	< LOQ [1]	-	76 (n=1)	-	-	-

LOQ[1]: Limit of quantification (lowest calibration level). Other mycotoxins (aflatoxin B1, B2, G1, G2; DON; fumonisin B1, B2; HT-2; ochratoxin A; T-2 toxin and roquefortine E) were below detectable limits in all samples. Mycotoxin results were calculated from positive samples. n =, Refers to the number of silages that tested positive for the mycotoxin and on which the mean is based. s.d.: Standard deviation.

black (0.9) or white (0.1) plastic stretch-film. Almost three-quarters (0.7) of the bales were wrapped at the site of baling prior to transfer to their storage location, with the remainder transported to their storage location before wrapping. Apart from being stored out of reach of livestock, half the bales had no additional protection, but the remaining bales were protected by fencing (0.2), bird netting (0.1), tyres (0.1) and paint (0.1). Bales were stored in single tiers at ground level (0.8) or stacked two tiers high (0.2), and were stored adjacent to sheds (0.5) or in a field (0.5).

Grass (0.85) silages accounted for most of the pit silages sampled, with the remainder being maize (0.15) silages. Over half the pit silages were harvested in May (0.3) and June (0.25) 2011, with the remainder in July (0.05), August (0.15), September (0.05) and October (0.2), and the latter were primarily maize. The majority (0.65) of pit silages had experienced less than 1 day of wilting, while the remaining silages had one (0.25) or two days of wilting (0.1). These silages were covered with two new sheets of black polythene film (0.9) or with one new and one old

Table 3: Summary statistics of the conventional chemical composition of pit farm silages collected in Co. Meath in 2012.

	Silage face		**3 m behind face**			
	(n=18)		**(n=18)**			
Conventional analysis	Mean	s.d.	Mean	s.d.	s.e.d.	*P value*
Dry matter (g/kg)	257	38.4	242	39.8	5.0	**
Dry matter digestibility (g/kg)	705	55.7	702	43.9	7.8	NS
Ash (g/kg DM)	83	33.8	83	23.0	4.0	NS
Crude protein (g/kg DM)	137	29.8	141	31.8	2.7	NS
pH	3.85	0.47	3.89	0.37	0.04	NS
Lactic acid (g/kg DM)	104	49.3	98	36.1	8.4	NS
Acetic acid (g.kg DM)	27	10.4	27	7.0	2.3	NS
Propionic acid (g/kg DM)	2.9	3.47	2.4	2.01	0.5	NS
Butyric acid (g/kg DM)	3.4	4.6	5	7.0	0.9	NS
Ethanol (g/kg DM)	16	8.0	17	8.7	1.2	NS
WSC (g/kg DM)	19	25.4	25	26.1	2.6	NS
Ammonia–N (g/kg N)	103	64.0	94	47.3	8.6	NS

P values relate to paired t-test for sampling location. s.d.: Standard deviation; WSC, water soluble carbohydrate;

Table 4: Summary statistics of the mycotoxin composition (µg/kg DM) of pit farms silages in Co. Meath in 2012.

		Silage face (n=18)				**3 m behind face (n=18)**				
Mycotoxin	**LOQ[1]**	**n =**	**Max**	**Mean**	**s.d.**	**n =**	**Max**	**Mean**	**s.d.**	**s.e.d.**	**P-value**
Andrastin A	50	1	863	863	-	2	500	304	277	24.1	P> 0.05
Beauvericin	5	-	<LOQ[1]	<LOQ[1]	-	1	21.8	21.8	-	-	P> 0.05
Enniatin A1	10	1	25	25	-	1	23.1	23.1	-	-	P> 0.05
Enniatin B	25	5	204	144	39	7	255	128	93	14.9	P> 0.05
Enniatin B1	25	1	66.3	66.3	-	1	80.9	80.9	-	-	P> 0.05
Mycophenolic acid	40	1	1419	1419	-	3	167	146	18	12.8	P> 0.05
Roquefortine C	40	2	1194	493	684	2	500	374	181	23.6	P> 0.05
Zearalenone	10	-	<LOQ[1]	<LOQ[1]	-	1	76.6	77	-	-	P> 0.05

[1]LOQ: Limit of quantification. P values relate to the Wilcoxon Signed ranked test accounting for sampling position; Summary statistics relate to positive samples only. n =, Refers to the number of silages that tested positive for the mycotoxin and on which the mean is based.
DM: Dry matter; s.d.: Standard deviation

sheet (0.1), and were weighted down with tyres. Just over half (0.55) of the horizontal pits had a clean, flat and even silage face, indicative of the use of a shear grab, while the other (0.45) silages had a relatively uneven and untidy appearance. The mean [standard deviation (s.d.)] time taken for a full face of silage to be removed was 2.8 (1.26) days. The mean (s.d.) daily air temperature during the month of February when this study was undertaken was 6.4ºC (3.19ºC).

Baled vs pit silages

Baled and pit silages summary statistics (Table 1) indicate that baled silages had higher (P< 0.01) DM, pH and WSC values, and lower (P<0.05) DMD, crude protein, lactic acid, acetic acid, propionic acid, ethanol and NH_3-N values compared with the pit silages. Of the 20 mycotoxins assayed, baled silages contained [mean of positive values (no. of values in mean)] mycotoxin concentrations (µg/kg DM) of beauvericin 36 (2), enn. A 9.3 (3), enn. A_1 54 (8), enn. B 351 (9), enn. B_1

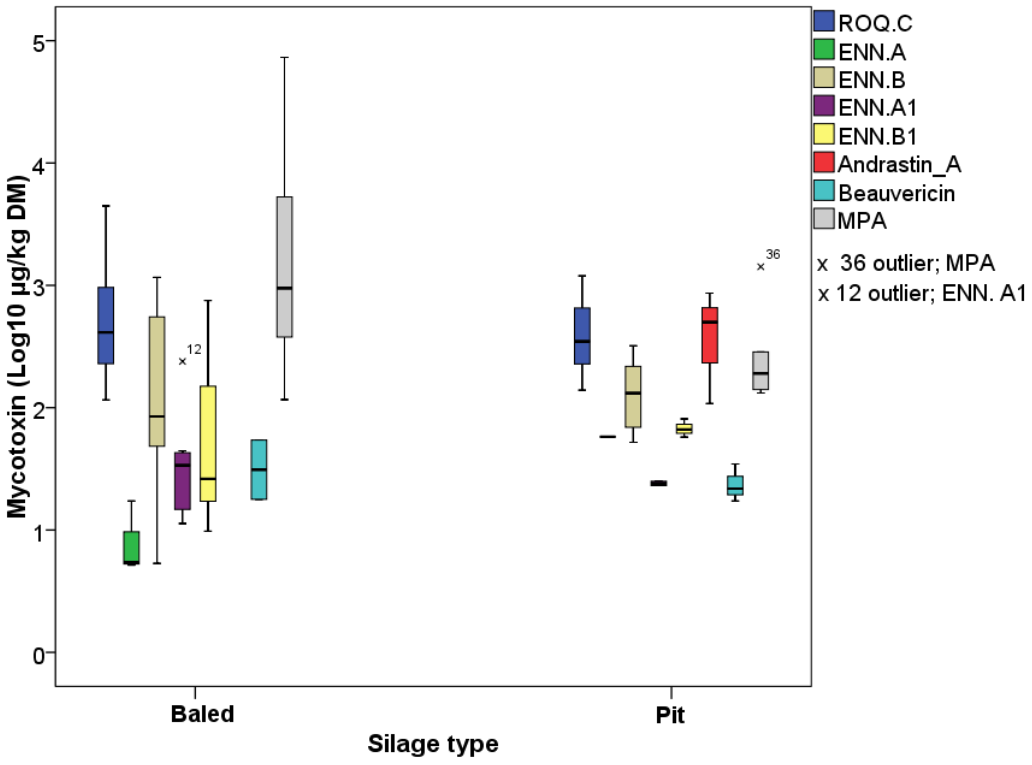

Fig. 3. Box plot graph of the mycotoxin profile detected in baled and pit silages collected from farms in Co. Meath in February 2012. ENN: Enniatin; MPA: mycophenolic acid; ROQ.C: Roquefortine C.

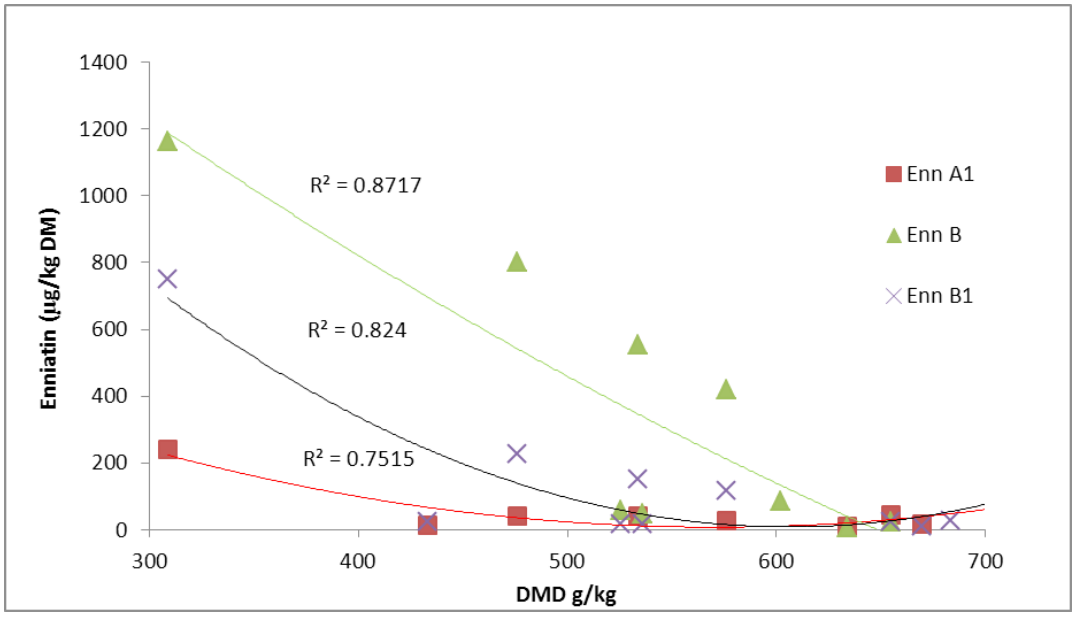

Fig. 4. Scatter plot graph depicting the relationship between the occurrence of enniatins and the dry matter digestibility of baled grass silages collected in Co. Meath in March 2012. DM: Dry matter; DMD: Dry matter digestibility; ENN: Enniatin.

136 (10), MPA 11,157 (8) and roquefortine C (Roq. C) 1037 (8) and pit silages contained beauvericin 25 (2) enn. A_1 18 (2), enn. B 194 (9), enn. B_1 57 (3), MPA 287 (6), Roq. C 3649 (6) and zearalenone 76 (1). Mycotoxin profiles are also displayed as a box plot graph (Fig. 3; $\log_{10}$ scale) that shows the ranges and means of the mycotoxins detected in each silage type. Mycotoxin results in Table 1 are not on a log scale, and are based on detectable mycotoxin positive samples only.

There was no significant difference in mycotoxin content between baled and pit silages. Correlations between mycotoxins and other chemical characteristics were assessed and there were no significant relationships except for enniatins and DMD. In addition, there was also no significant correlation between wilting and any mycotoxin occurrence. The occurrence of enniatins and their relationship with DMD in baled silages is represented in Fig. 4 as a scatter plot, and the R^2 values ranged from 0.75 to 0.87.

Sampling position in pit silos

Tables 2 and 3 outline summary statistics for chemical and mycotoxin composition, respectively, for sampling position in farm pit silos. Silage sampling position affected DM content ($P<0.01$) alone among conventional silage composition variables, and had no effect (>0.05) on mycotoxin composition.

Discussion

Conventional chemical characteristics of baled and pit silages.

In agreement with similar comparisons of silages produced in Co. Meath (McEniry *et al.,* 2007) and nationally (Keating and O'Kiely, 1997 a, b), silage DM concentration values indicate that herbages for both baled and pit silages were wilted, with the wilting of herbage for baled silages being more extensive. This in turn resulted in baled silages having a more restricted fermentation as evidenced by their lower concentrations of fermentation products and their higher pH and WSC values compared with the pit silages. Thus, for example, Keating and O'Kiely (1997a, b) assembled a national summary of baled (n= 853) and pit (n=15,530) silages on Irish farms and reported mean (s.d.) DM and pH values for baled silages of 324 (141.1) g/kg and 4.8 (0.62), with corresponding values for pit silages of 216 (48.7) g/kg and 4.0 (0.34), respectively.

The fermentation products in baled silages were less dominated by lactic acid, indicating a relatively larger influence from micro-organisms other than obligatory homofermentative and facultatively heterofermentative lactic acid bacteria. The different microbiome in baled and pit silages was demonstrated by McEniry *et al.,* (2006) who enumerated higher colony counts of yeast and Clostridia, lower counts of bacilli and comparable numbers of total lactic acid bacteria and enterobacteria on baled compared to pit silages. On average, in this current study, both silage types were satisfactorily preserved as assessed by the criteria proposed by Haigh and Parker (1985), and showed limited evidence of undesirable secondary fermentations. The baled and pit silage history and management practices recorded largely reflect those also observed in other regional or national surveys (McEniry *et al.,* 2006; O' Brien *et al.,* 2005).

The higher DMD and CP in pit silages indicate that they were made from herbages harvested at a less advanced stage of maturity than the herbages used for producing baled silages. This difference between silage types agrees with McEniry *et al.,* (2006).

Mycotoxins profiles in baled and pit silages

Currently the mycotoxins that are regulated in EU feedstuffs are aflatoxin B1 (maximum of 20 µg/kg in all feed materials, although lower thresholds exist for complete feedingstuffs), DON (maximum guidance value of 8000 µg/kg in cereal and cereal products), fumonisins B1+B2 (maximum guidance value of 50,000 µg/kg for B1 and B2 together in feed for adult ruminants), OTA (maximum guidance value of 250 µg/kg in cereal and cereal products) and zearalenone (maximum guidance value in complete feedstuffs for calves, dairy cattle, sheep and goats of 500 µg/kg). The European feed industry is currently in the process of developing industry guidance on Recommendation 2013/165/EU for T-2 and HT-2 (EU Commission, 2013). Zearalenone was the only one of the eight regulated mycotoxins found in this study.

Silages can be contaminated by metabolites and their derivatives from both pre-harvest and post-harvest moulds. This contamination can vary extensively year on year, majorly affected by climate (Magan *et al.,* 2011). Common pre-harvest moulds of silage crops include *Fusarium, Alternaria* and *Aspergillus* while common post-harvest moulds include *Penicillium*, *Zygomycetes, Aspergillus fumigatus* and *Byssochlamys nivea* (Cheli *et al.*2013; Storm *et al.* 2008). Among baled silages on Irish farms, O'Brien *et al*. (2005) identified *P. roqueforti, Mucor* and *Geotrichum* as the most common moulds. Furthermore, 79 isolates of *P. roqueforti* and 78 isolates of *P. paneum* collected from baled silages were capable of producing an extensive and diverse range of secondary metabolites *in vitro* (O'Brien *et al.,* 2006).

In the present study, each silage type contained mycotoxins capable of being produced by both *Fusarium* and *Penicillium* moulds, thereby suggesting the effects of both pre- and post-harvest moulds, respectively (Table 1; Fig. 3). However, even though baled and pit silages had generally similar mycotoxin

profiles (Fig. 3), baled silages tended to have a greater range of mycotoxin concentrations.

MPA, a post-harvest *Penicillium* metabolite, was found in both pit and baled silages. One particular baled silage sample had an MPA concentration of 73,118 µg/kg DM, which is higher than values reported by Driehuis *et al.* (2008), Schneweis *et al.* (2000) or Storm *et al.* (2014) and strongly affects the mean value for MPA in baled silages. Almost half of the baled silages and one-third of pit silages contained detectable concentrations of MPA, and the mean values were numerically lower for the pit silages [393 (pit) vs 11,157 (bale) µg/kg DM]. In a survey of grass and maize silages in Germany, Schneweis *et al*. (2000) found MPA in 32% of samples, with individual MPA positive values ranging from 20 to 35,000 (mean of positive values 1400) µg/kg DM. There is limited information available about the toxicological effects of MPA on livestock. High MPA concentrations exert potential immunosuppression effects and consumption of immunosuppressive compounds increases the risk of infectious diseases in livestock (Mohr *et al.,* 2007). Exposure of cattle to MPA has previously resulted in ruminal dysbacteriosis and ruminitis due to its antimicrobial effects (Kopp-Holtwiesche and Rehm, 1990). There are limited data available on the carryover of MPA into animal products, although the likelihood of carryover is low, because in studies with sheep, a low transfer of MPA to blood plasma and body tissues was detected (Tuller, 1998; Mohr *et al.,* 2007).

Roq. C commonly co-occurred with MPA in silages. This co-occurrence was observed in baled silage always and for pit silages, half of the time. . The concentrations of Roq. C were similar to values recorded in farm silages in other studies (Driehuis *et al.,* 2008; Auerbach *et al.,* 1998; Tuller *et al.,* 1998). The concentrations of Roq. C determined in this study should not give rise for concern, since Tuller *at al*., (1998) determined that dosages up to 25,000 µg/kg DM had no toxicological relevance to sheep. A Swedish study, on the other hand, established a relationship in a herd of dairy cows between their disease symptoms (including paralysis, ketosis and inappetence) and the presence of Roq. C at a high concentration (25,000 µg/kg) in the feed consumed, and attributed this to an extensive infection by *P. roqueforti* mycelia (Haggblom, 1990). Nevertheless, the concentrations determined in the present study are much lower, suggesting little cause for concern.

The trend towards higher numerical values for the *Penicillium* mycotoxins MPA and Roq. C within baled silages agrees with the high incidence of *P. roqueforti* identified in baled silages by O'Brien *et al.,* (2005), and suggests that excess air had penetrated the plastic stretch-film barrier in which these bales were wrapped. Baled silage is usually wrapped in a thinner layer of plastic film to exclude air and has a higher silage surface area to total volume ratio than pit silage (Forristal and O'Kiely, 2005). This, together with the real risk of damage to the integrity of this plastic seal during handling and storage of the wrapped bale, makes it more susceptible to mould growth and thus contamination with mycotoxins produced by post-harvest moulds.

Zearalenone, a potent, non-steroidal oestrogen that can cause abortions and fertility problems in livestock (Zinedine *et al*., 2007), and the only EU regulated mycotoxin found among these silages, was detected in a single pit of grass silage at 77 µg/kg DM (4% of the EU threshold value). This compares to the average concentration of 80 µg/kg zearalenone in animal feeds (maize, wheat, barley, soybean meal, corn gluten meal, dried distillers grains with solubles, straw, silage and finished feed) in Europe (2013–2014) (www.biomin.net 2014). It was concluded that the level of risk associated with this average concentration is low for ruminants. Therefore, the risk posed from zearalenone in the single pit of silage in this study appeared to be low. In addition, EFSA has summarised and evaluated studies of the carryover of zearalenone from animals to humans in the food chain and concluded that there is only limited deposition of zearalenone in meat and that the transfer to milk is also low (EFSA, 2004).

Enniatins and beauvericin were also detected in baled and pit silages (Table 1, Fig. 3, Fig. 4,). These *Fusarium* mycotoxins are ionophores and are considered to originate pre-harvest. Beauvericin and enniatins have been detected in studies of maize silages, but at generally higher concentrations of 5000–60,000 µg/kg DM and 25–200 µg/kg fresh weight, respectively (Zachariasova *et al.,* 2014, Logricco *et al.,* 2002). Studies on the effects of enniatins or beauvericin in livestock are limited, although mice administered massive oral doses of beauvericin did not exhibit clinical symptoms (LD50 >100 mg/kg; Omura *et al.,* (1991) cited in Jestoi, (2008). Oral doses between 6000 and 50,000 µg enniatins/kg bodyweight (BW) in mice, rats, guinea pig and rabbits produced no toxic findings (Jestoi, 2008). Thus, for example, if a 600 kg bovine consumes 17 g silage DM/kg BW, then the challenge posed by enniatins (combined) in baled silage from this study would be 9.6 µg/kg BW. This is considerably less than the quantity used by Jestoi (2008), suggesting that the risk from the enniatins from these silages was relatively low.

There was a general inverse relationship observed (Fig. 4) between the occurrence of enniatins and the *in vitro* DMD values in baled silages. The root of this relationship is unclear; however, it is possible that (a) enniatins were more prevalent on less digestible herbage that, for example, may also contain a higher proportion of low DMD dead vegetation pre-harvest; (b) the enniatins have interfered with the activity of the rumen microbial inoculum used in the *in vitro* DMD assay; or (c) *Fusarium* has utilized the more digestible substrate within

pre-harvest crop or post-harvest silage, reducing overall digestibility as well as producing enniatins.

Sampling position in pit silos

It was expected that, on average, across all 18 pit silos, silage sampled from 3 m behind the silage face would have completed their fermentation and be in a stable anaerobic phase as described by McDonald *et al.,* (1991). The expectation for silage at the silage face was different because of its direct exposure to air, and it was expected that this exposure would permit respiration to commence. If the latter was sufficiently extensive it would have been evidenced by a reduction in silage lactic acid and WSC concentrations and DMD, and an increase in pH and temperature, and possibly ash and storage mycotoxins. Since sampling position did not have a significant effect on any of these variables, the explanation is either: (a) silage 3 m behind the feed face deteriorated as extensively as at the feed face, or (b) the silage at the feed face remained as stable as the silage at 3 m behind the feed face. The dominance of lactic acid in the fermentation products, the normal concentrations of WSC and ash and the relatively low concentrations of storage mycotoxins suggest that the latter scenario prevailed. In addition, extensive heating at the silage face, although not measured, was not noticed.

Although there was little evidence of marked aerobic deterioration at the silage feed face, it would be surprising if no deterioration occurred in silage exposed to air. The low extent of aerobic deterioration evident in the samples taken from the silage feed face likely reflects:

(a) The presence of a relatively high content of water in the silages. Muck *et al.,* (2003) explained that increasing silage water content restricts access to oxygen for spoilage micro-organisms by reducing silage porosity. They also indicated that it reduces the temperature rise produced by the heat generated by aerobic microbial growth because of the high specific heat capacity of water. Restricting temperature rise has the effect of slowing microbial activity.

(b) The relatively short duration of the silage feed face being exposed to air, since the full feed face was removed for feeding on average every 2.8 days.

(c) Mean ambient air temperatures of only 6.4ºC at the times the silages were sampled. For example, O'Kiely *et al*. (1989) measured DM losses during the aerobic exposure of samples from 84 farm silages and recorded mean losses of 26 and 269 g/kg when they were aerobically stored for 10 days at 10 and 25ºC, respectively.

(d) The silage cores taken at the feed face provided samples to a depth of 0.6 m. This depth was chosen to approximate the depth of silage blocks typically removed by mechanical unloaders, including shear grabs. However if aerobic deterioration was restricted to, for example, the outer 1 cm of exposed silage then its impact could have been quite diluted by non-deteriorated silage in the remainder of the core sample. Furthermore, that storage mycotoxins (Roq. C, MPA and andrastin A) were numerically but not significantly higher at the feed face hints at some mould activity at that location.

While mean pH values at both sampling points were below 3.9, this itself might not markedly restrict yeast or mould growth under aerobic conditions. Muck *et al.,* (1991), in modelling aerobic fungal growth in silage, concluded that the attainment of a low pH does not necessarily restrict yeast or mould growth, and therefore need not restrict aerobic deterioration.

The higher DM concentration at the silage face than 3 m behind it was unexpected given that sampling position had no effect on the other variables. However, the scale of the difference in DM concentration was relatively small. Potential explanations for the difference include (a) drying of the feed face due to exposure to lower humidity ambient air, (b) drying of the feed face due to heat generated by respiration at the aerobic feed face, or (c) removal of juice from the silage, due to squeezing as the coring apparatus received the sample. Ambient drying is not a plausible explanation as this would require considerable influx and efflux of air through the silage and this would likely have stimulated aerobic deterioration. The difference in silage DM concentration was unlikely to be due to respiration since the other characteristics of the substrate should then have also differed. It is unlikely it was due to the coring process squeezing silage juice as this should have also impacted on the concentrations of solutes such as fermentation products and WSC. Therefore, the cause of the small difference detected in DM concentration is not evident. It is noteworthy that when McEniry *et al.,* (2006) compared the composition of the outer 20 cm with the next inward 40 cm of silage from baled silages, no difference in DM concentration was detected.

Whereas, this study analysed silages from two positions within the pit silos (pit face and 3 m behind the pit face), the findings of Storm *et al.,* (2010) allow us to expect that this result would hold over a longer timeframe, as they analysed maize silages over an 8-month period across five time points and found no difference in chemical composition or microbiological profiles.

Conclusion

Despite the differences in conventional chemical compositional traits between baled and pit silages, no significant difference in the incidence or concentration of mycotoxins between these silage types were evident. Overall, mycotoxin concentrations were generally relatively low.

Within pit silages, sampling position (silage feedout face vs 3 m behind feedout face) affected neither conventional chemical compositional traits (except DM) nor the incidence or concentration of mycotoxins.

The concentrations of individual mycotoxins taken at the feed face within pit silos or bales approximate to what was offered to livestock at the time of sampling on these 38 farms in Co. Meath, and were below EU guideline thresholds (EU Commission, 2002; 2006). However, caution needs to be exercised in concluding on the overall mycotoxin challenge since the effects of mixtures of mycotoxins can be more severe than the sum of their individual effects (Šegvić Klarić, 2012).

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References

AOAC. 1990. "Official methods of Analysis". First supplement (1990) to the 15th edition, method 990-03. AOAC, Arlington, VA, USA.

Auerbach, H., Oldenburg, E., Weissbach, F. 1998. Incidence of Penicillium roqueforti and roquefortine C in silages. *Journal of the Science of Food and Agriculture* **76**: 565–572.

Binder, E.M. 2007. Managing the risk of mycotoxins in modern feed production. *Animal Feed Science and Technology* **133**: 149–166.

BIOMIN. (2014) *Biomin world mycotoxin survey* [Online] August 2014. Available from: www.biomin.net/en/appwebversion/survey/#.UyFoeFF5Nqk [Accessed: 20th August 2014].

CENTRAL STATISITICS OFFICE. (2007) *Crops and livestock survey.* [Online] June 2007. Available from: http://www.cso.ie/en/media/csoie/releasespublications/documents/agriculture /2007/croplsfinal_june2007.pdf [Accessed: 6th June 2008]

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

Driehuis, F., Spanjer, M.C., Scholten, J.M., te Giffel, M.C., 2008. Occurrence of Mycotoxins in Feedstuffs of Dairy Cows and Estimation of Total Dietary Intakes. *Journal of Dairy Science* **91**: 4261–4271.

EFSA, 2004 (a). Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to zearalenone as undesirable substance in animal feed. *The EFSA Journal* **89:** 1-35

EFSA, 2004 (b) Opinion of the scientific panel on contaminants in food chain on a request from the commission related to aflatoxin B_1 as undesirable substance in animal feed. *The EFSA Journal* **39:** 1-37.

EFSA, 2006. Opinion of the scientific panel on contaminants in food chain on a request from the commission related to ochratoxin A as undesirable substance in food. *The EFSA Journal* **365**: 1-56.

E.U Commission. 2002. Directive 2002/32/EC of the European parliament and of the council of 7 May 2002 on undesirable substances in animal feed 2002/32/EC. *Official Journal of the European Union* 2002:32

E.U Commission. 2006. Commission recommendation (EC) No. 576/2006 of 17 August 2006 on the presence of deoxynivelenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. *Official Journal of the European Union 2006:576*

E.U Commission, 2013. Commission recommendation (EC) No. 165/2013 of 23 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products. *Official Journal of the European Union 2013:165*

Forristal, P.D., O'Kiely, P., 2005. Baled silage: an overview of its development and stretch wrapping needs. In: "Silage Production and Utilization". *Proceedings of the XIV International Silage Conference, Belfast,* pages 83–96.

Haigh, P.M., Parker, J.W.G., 1985. Effect of silage additives and wilting on silage fermentation, digestibility and intake, and on live weight change of young cattle. *Grass and Forage Science* **40:** 429–436.

Häggblom, P., 1990. Isolation of roquefortine C from feed grain. *Applied Environmental Microbiology* **56:** 2924–2926.

Jestoi, M., 2008. Emerging *Fusarium* mycotoxins Fusaproliferin, beauvericin, enniatins, and Moniliformin -a review. *Critical Reviews in Food Science and Nutrition* **48:** 21–49.

Keating, T., O'Kiely, P., 1997 a. Big bale silage quality 1993-1996. *Proceedings of the Agriculture Research Forum*, *Tullamore,* pages 121–122.

Keating, T., O'Kiely, P., 1997 b. Irish Farm silage 1993-1996. *Proceedings of the Agriculture Research Forum*, *Tullamore,* pages 123–124.

Kopp-Holtwiesche, B., Rehm, H.J., 1990. Antimicrobial action of roquerfortine. *Journal of Environmental Pathology, Toxicology and Oncology* **10:** 41–44.

Logrieco, G. Mulé, A. Moretti, A. Bottalico, 2002. Toxigenic Fusarium Species and Mycotoxins Associated with Maize Ear Rot in Europe. *European Journal of Plant Pathology* **108:** 597–609

Magan, N., Medina, A., Aldred, D., 2011. Possible climate-change effects on mycotoxin contamination of food crops pre- and post-

harvest: Mycotoxins and climate change. Plant Pathology 60, 150–163

McDonald, P., Henderson, N., Heron, S., 1991. "The biochemistry of silage", Chalcombe, Marlow, England, 340 pages.

McElhinney, C, O'Kiely, P., Elliot, C., Danaher, M., 2013. Development of a Method for the Detection of 20 Mycotoxins in silage by UHPLC-MS/MS, in: Emerging Risks in the Feed Supply Chain. Presented at the Biotoxins Knowledge Network Annual Conference, Belfast, N. Ireland, p. 25.

McEniry, J., O'Kiely, P., Clipson, N.J.W., Forristal, P.D., Doyle, E.M., 2006. The microbiological and chemical composition of baled and precision-chop silages on a sample of farms in County Meath. *Irish Journal of Agricultural and Food Research* **45:** 73–83.

McEniry, J., O'Kiely, P., Clipson, N.J.W., Forristal, P.D., Doyle, E.M., 2007. The relative impacts of wilting, chopping, compaction and air infiltration on the conservation characteristics of ensiled grass. *Grass and Forage Science* **62:** 470–484.

Moeser, S., (2001) *Influence of zearalenone and zeranol in heifers.* A thesis submitted in partial fulfillment of the requirements of University of Veterinary Medicine, Vienna for the Degree of Doctor of Philosophy. Vienna.

Mohr, A.I., Lorenz, I., Baum, B., Hewicker-Trautwein, M., Pfaffl, M., Dzidić, A., Meyer, H.H.D., Bauer, J., Meyer, K., 2007. Influence of oral application of mycophenolic acid on the clinical health status of sheep. *Journal of veterinary medicine. A, physiology, pathology, clinical medicine* **54:** 76–81.

Muck, R.E., Pitt, R.E., Leibensperger, R.Y., 1991. A model of aerobic fungal growth in silage. 1. Microbial characteristics. *Grass and Forage Science* **46:** 283–299.

Muck, R.E., Moser, L., Pitt, R.E., 2003. Postharvest factors affecting ensiling, in: "Silage Science and Technology". American Society of Agronomy : Crop Science Society of America : Soil Science Society of America, Madison, Wisconsin, USA, Pages 251-304.

O'Brien, M., O'Kiely, P., Forristal, P.D., Fuller, H.T., 2005. Fungi isolated from contaminated baled grass silage on farms in the Irish Midlands. *FEMS Microbiology Letters* **247:** 131–135.

O'Brien, M., Nielsen, K.F., O'Kiely, P., Forristal, P.D., Fuller, H.T., Frisvad, J.C., 2006. Mycotoxins and Other Secondary Metabolites Produced in Vitro by *Penicillium* paneum and Penicillium roqueforti isolated from baled grass silage in Ireland. *Journal of Agricultural and Food Chemistry* **54:** 9268–9276.

O'Kiely, P., Forristal, P.D., Lenehan, J.J., Fuller, H., Brady, K., Barlow, M., 1998. Characteristics of baled silage on Irish farms. *Farm Foods* **1:** 29–32.

O'Kiely, P., 1989. Aerobic stability of farm silages. *Irish Journal of Agricultural Research* **28:** 102–103.

Porter, M.G., Murray, R.S., 2001. The volatility of components of grass silage on oven drying and the inter-relationship between dry-matter content estimated by different analytical methods. *Grass and Forage Science* **56:** 405–411.

Schneweis, I., Meyer, K., Hörmansdorfer, S., Bauer, J., 2000. Mycophenolic acid in silage. *Applied environmental microbiology*. **66:** 3639–3641.

Šegvić Klarić, M., 2012. Adverse Effects of Combined Mycotoxins. *Archives of Industrial Hygiene and Toxicology* **63**:4

Storm, I.M.; Sørensen, J.L.; Rasmussen, R.R.; Nielsen, K.F.; Thrane, U., 2008. Mycotoxins in silage. *Stewart Postharvest Review 4*.

Storm, I.M.L.D., Kristensen, N.B., Raun, B.M.L., Smedsgaard, J., Thrane, U., 2010. Dynamics in the microbiology of maize silage during whole-season storage. *Journal of Applied Microbiology* **109:** 1017–1026.

Storm, I., Rasmussen, R., Rasmussen, P., 2014. Occurrence of Pre- and Post-Harvest Mycotoxins and Other Secondary Metabolites in Danish Maize Silage. *Toxins* **6:** 2256–2269.

Tilley, J.M.A., Terry, R.A., 1963. A two-stage technique for the *in vitro* digestion of forage crops. *Grass and Forage Science* **18:** 104–111.

Thomas, A., T., 1977. An automated procedure for the determination of soluble carbohydrates in herbage. *Journal of the Science of Food and Agriculture* **28:** 639–642.

Tüller, G., Armbruster, G., Wiedenmann, S., Hänichen, T., Schams, D., Bauer, J., 1998. Occurrence of roquefortine in silage - toxicological relevance to sheep. *Journal of Animal Physiology and Animal Nutrition* **80**: 246–249.

Zachariasova, M., Dzuman, Z., Veprikova, Z., Hajkova, K., Jiru, M., Vaclavikova, M., Pospichalova, M., Florian, M., Hajslova, J., 2014. Occurrence of multiple mycotoxins in European feeding stuffs, assessment of dietary intake by farm animals. *Animal Feed Science and Technology*. **193:** 124-140

Zinedine, A., Soriano, J.M., Moltó, J.C., Mañes, J., 2007. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. *Food and Chemical Toxicology* **45:** 1–18.