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OCCURRENCE OF MYCOTOXINS IN CONSERVED GRASS AND LEGUME FORAGES – A SYSTEMATIC REVIEW

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Abstract

Conserved forages are important feed for livestock and equines all over the world. However, contamination with mycotoxins may occur, leading to health issues to the animals and to humans that consume products of animal origin. As information on the occurrence of mycotoxins in conserved grass and legume forages is scarce, a review of the literature published between January 1992 and July 2022 was performed to assess the worldwide occurrence and levels of mycotoxins in hay, haylage, and silage. Zearalenone (ZEN), deoxynivalenol (DON), and aflatoxins (AFs) were the most common mycotoxins found in conserved forage in North America, Africa, and the Middle East, with ZEN and AFs in levels above the maximum levels determined in European Union (EU) regulations for animal feed. Ochratoxin A (OTA) was detected in high levels in conserved forages in Europe. Although other mycotoxins were found, they were detected in lower frequencies and levels. Co-occurrence of mycotoxins was identified in more than half of the studies analyzed. There were few studies carried out in Asia, Middle East, Oceania, and no information was available on Central and South America, reinforcing the need of studies on the issue in these regions.

Key words: feed safety, animal feed, mycotoxins, hay, haylage, silage

Fresh and conserved forages are important in the nutrition of livestock and equines. These feeds, however, may be contaminated by fungi and mycotoxins in the field or during storage (Mostrom and Jacobsen, 2020; Müller, 2018; Ogunade et al., 2018). Mycotoxins, secondary metabolites of low molecular weight produced by filamentous fungi, are a food and feed safety concern due to their deleterious effects on the health of humans and animals (Kemboi et al., 2020; Tolosa et al., 2021).

Mycotoxigenic fungi are ubiquitous, and the production of these metabolites is influenced by genetic and environmental factors (Buszewska-Forajta, 2020; Gajęcki et al., 2020; Tolosa et al., 2021). Currently, there are more than 400 mycotoxins identified, although the most concerning regarding the human and animal health are the widely regulated aflatoxins (AFs), fumonisins (FBs), ochratoxin A (OTA), zearalenone (ZEN) and deoxynivalenol (DON) (Buszewska-Forajta, 2020). Other non-regulated mycotoxins, sometimes reported as emerging mycotoxins such as beauvericin (BEA) and enniatins (ENNs), have gained prominence in recent years because of their frequent occurrence in food and feed products (Logrieco et al., 2018).

Mycotoxicosis can develop acutely or chronically, and the main signs are immunosuppression, hepatotoxicity, nephrotoxicity, neurotoxicity, digestive alterations, carcinogenesis, and teratogenesis, and the toxicity can vary depending on the age, species and animal category (Buszewska-Forajta, 2020; Gajęcki et al., 2020; Mahato et al., 2021). Young animals are more susceptible to the effects of mycotoxins, and horses are more sensitive than ruminants, which are generally more resistant than monogastric animals due to the ability of their rumen microbiota to degrade and inactivate some mycotoxins (Andersen et al., 2020; Adegbeye et al., 2020; Gallo et al., 2015; Kemboi et al., 2020). Although intoxicated animals at low exposure doses of mycotoxins not always develop clinical signs compatible with mycotoxicosis, they may show a reduction in productivity leading to economic losses and the risk of transmission of mycotoxins through animal products (Adegbeye et al., 2020; Buszewska-Forajta, 2020; Gallo et al., 2015; Manni et al., 2022).

Forage conservation is an important strategy in animal production all over the world. In some regions of the globe, this type of feed corresponds to 50–70% of the dry matter ingested daily by animals. Conserved forages are especially necessary during periods of scarcity, when there is a significant decrease in the availability of fresh forage and in the nutritional quality of the pastures. At these moments, conserved forages contribute to maintain production indices and to reduce animal mortality (Gallo et al., 2015; González-Jartín et al., 2022; Juan et al., 2020; Kemboi et al., 2020; Rodríguez-Blanco et al., 2021).

The most common forage conservation techniques use drying and ensiling, hay, haylage, and silage making. In hay production, forage moisture levels will be reduced to less than 40%. Because of this, hay may be stored in aerobic conditions. Haylage making leads to forage with intermediate moisture levels (40–60%) that is kept in wrapped bales, creating an anaerobic environment. Silage has higher moisture levels, above 60%, and is kept in closed silos that also create an anaerobic environment, but at higher fermentation rates, which lead to lower pH (Müller, 2018; Rodríguez-Blanco et al., 2021; Schenck et al., 2019). Although efficient, errors in the production, storage, or handling of these conserved forages may enable growth of filamentous fungi and production of mycotoxins. Some fungi may even grow under the unfavorable anaerobic or microaerobic conservation conditions, such as *Aspergillus fumigatus*, *Penicillium roqueforti*, *P. paneum*, and *Fusarium oxysporum* (Andersen et al., 2020; Buszewska-Forajta, 2020; González-Jartín et al., 2022).

Mycotoxin occurrence and levels of contamination in corn silage or silage made of other cereals have been well documented in the literature. However, there is little information on the occurrence of mycotoxins in other types of conserved forages, such as hay, haylage, and grass and legume silage (Ceniti et al., 2021; Manni et al., 2022; Ogunade et al., 2018; Schenck et al., 2019). From a food safety standpoint, knowledge on the occurrence of mycotoxins in conserved forages is especially important in food-producing animals, as the amount of toxin consumed may influence their deposition and excretion rates in animal products, such as meat, milk and meat/dairy products (Adegbeye et al., 2020; Buszewska-Forajta, 2020; Manni et al., 2022).

Given the importance of grass and legume forages for the maintenance of animal production all over the world, the lack of knowledge on the occurrence of mycotoxins in these products, and the food safety impacts of levels of mycotoxins in the production chain of animal products, the objective of systematic review was to evidence the worldwide occurrence and levels mycotoxins in grass and legume forages conserved as hay, haylage, and silage.

Material and methods

Search strategy

A systematic review of the literature was carried out in three databases: PubMed, Scopus, and Web of Science, from January 1992 to July 2022 in the search for relevant articles on the occurrence of mycotoxins in grass and legume forages conserved as hay, haylage, and silage. The search strategy was based on the Cochrane protocol and the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, as shown in Figure 1 (Higgins and Green, 2011; Moher et al., 2015). The following combination of keywords was used: "mycotoxins" AND "forage" OR "fodder" OR "hay" OR "haylage" OR "silage" AND "grass" OR silage AND "legumes". Articles found were stored in the cloud of the database of EndNote Web (Clarivate Analytics), which enabled the automatic removal of duplicated articles. Duplicated articles that were not identified by the software were manually removed in a second step. After that, titles and abstracts of relevant articles were screened. Full texts were downloaded and analyzed following this initial screening.

Relevant articles were selected for eligibility, and the others were removed according to the following exclusion criteria: (1) articles with no original data, including reviews, systematic reviews, books, theses, and workshops; (2) studies on other mycotoxins, or reports on other products; (3) articles about the development of analytical methods only, or comparison of different analytical methods; (4) lack of specific data on mycotoxin production under natural and uncontrolled conditions. Lastly, eligible articles were carefully analyzed to be included, according to the following inclusion criterion: original descriptive articles with specific data on the occurrence and concentration of mycotoxins in grass and legume forages conserved as hay, haylage, and silage, without mixing with traditional grains and cereals, such as corn, sorghum, wheat, oat, rye, barley, and others. After the evaluation process was finished, 4,298 articles were excluded, and 28 articles met the inclusion criteria and were included in this review.

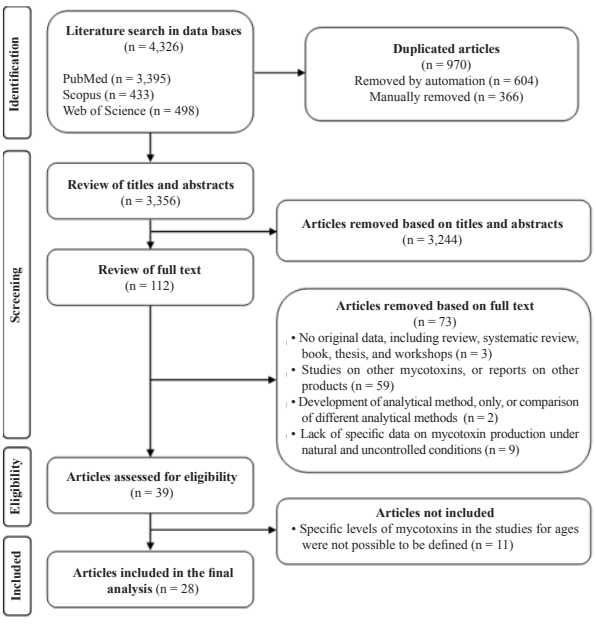


Figure 1. Search and data collection strategy with inclusion and exclusion criteria, based on the PRISMA guidelines (Moher et al., 2015)

Results

The available data on the occurrence of mycotoxins in grass and legume conserved forages in scientific reports published until July 2022 are presented in Table 1.

From the 28 articles included in this review, 20 of them were carried out in Europe, four in the Middle East, two in North America, one in Africa and one in Oceania. Except for studies carried out in Turkey or the Middle East, considered intercontinental regions, no study was identified in Asia. South and Central America studies were also lacking. The 28 articles comprised 34 studies on the occurrence of mycotoxins in conserved forages: 13 in hay, five in haylage, and 16 in silage. Nine studies tested small numbers of samples (<10 samples) of grass or legume forages, and none of the evaluated articles compared the mycotoxin occurrence in these types of materials.

Different analytical methods with various limits of detection (LOD) were employed in the quantification of mycotoxins in the studies analyzed, in the following order: liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), enzyme-linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC), and gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) (41, 32, 29 and 6% of the studies, respectively). However, in 11 studies, the LOD of the analytical method was not informed. GC-MS/MS and LC-MS/MS were the preferable techniques used for multi-mycotoxin analysis (MMA). In 91% of the evaluated studies, the presence of at least one mycotoxin was observed, while 62% of them reported the co-occurrence of mycotoxins.

Mycotoxins that were most found in grass and legume conserved forages were ZEN, DON, and AFs, which were detected in 60, 54 and 50% of the studies that evaluated those contaminants. In hay, the most frequent mycotoxins were DON (100%), AFs (78%), and ZEN (75%); in haylage, ZEN (40%); and in silage, ZEN (60%), DON (53%), and AFs (44%), in the studies in which they were tested.

Table 1. Occurrence of mycotoxins in grass and legume conserved forages reported from January 1992 to July 2022

Country	Types of material	*N*	Types of mycotoxins	Positive samples		Concentration		LOD (µg/kg)	Analytical method	Reference
				N	%	range (µg/kg)	mean (µg/kg)			
1	2	3	4	5	6	7	8	9	10	11
					Hay					
Canada	Grass and legumes	10	ZEN DON T-2	7 10 9	70 100 90	250−1,210 1,200−3,600 300−400	422 2,140 344.4	250 150 NR	ELISA	Raymond et al. (2000)
Germany	NR	28	ZEN DON NIV	12 4 2	43 14 7	NR−115 NR−69 NR−222	24 41 131	1 7 14	GC-MS/MS HPLC	Schollenberger et al. (2006)
Ireland and Canada	NR	109	ZEN	14	13	NR	NR	NR	ELISA	Buckley et al. (2007)
Australia	Grass and legumes	2	AFB_1 ZEN DON	2 2 1	100 100 50	6−13 1.2−5.81 160	9.5 3.5 160	1 32 50	HPLC	Reed and Moore (2009)
Serbia	Grass and legumes	16	AFs	NR	NR	NR	2.65	NR	ELISA	Grdović et al. (2010)
France	Grass and legumes	27	MMA for 15 metabolites	ND	–	–	–	15−75	LC-MS/MS	Seguin et al. (2010)
Czech Republic and United Kingdom	NR	4	FB_1 FB_2 ZEN DON NIV AOH MPA RC ENNs BEA	4	100	NR−190 a NR−36 a NR−91 a NR−62 a NR−224 a NR−12 a NR−3,236 a NR−746 a NR−845 a NR−128 a	35 a 26 a 37 a 46 a 116 a 5 a 428 a 69 a 56.5 a 47 a	NR	LC-MS/MS	Zachariasova et al. (2014)
Greece	Legumes	42	AFG_2 OTA	9 7	21 17	4−87 12−1,697	23.8 276.6	3	LC-MS/MS	Tsiplakou et al. (2014)
Iran	Legumes	44	AFB_1	43	98	0−66.57	6.75	1	ELISA	Hashemi (2016)
Pakistan	NR	8	AFB_1	1	13	0.56−22.31	4.91	0.02	ELISA	Chohan et al. (2016)
Iran	Legumes	40	AFB_1 AFB_2 AFG_1 AFG_2	24 24 10 8	60 60 25 20	0.12−0.48 0.015−0.38 0.05−0.18 0.03−0.09	0.2 0.13 0.06 0.03	0.12 0.015 0.05 0.03	HPLC	Bahrami et al. (2016)
Italy	NR	48	AFB_1	45	94	2.1−7.7	3.35	1−40	ELISA	Ceniti et al. (2021)
Qatar	NR	8	ZEN	8	100	2.01−22.8	8.72	NR	ELISA	Zeidan et al. (2022)

Table 1 – contd.

1	2	3	4	5	6	7	8	9	10	11
					Haylage					
Ireland	NR	40	ZEN	3	7	NR	NR	NR	ELISA	Buckley et al. (2007)
France	Grass	8	MMA for 15 metabolites	ND	–	–	–	15−75	LC-MS/MS	Seguin et al. (2010)
Sweden and Norway	Grass and legumes	100	ZEN DON T-2 HT-2 AOH ENNs BEA	1 12 2 4 8 14 10	1 12 2 4 8 14 10	8 b 69−479 b 8−11 b 19−78 b 11−1,452 b 10−283 b 11−988 b	8 b 238 b 10 b 35 b 212 b 56 b 248 b	5 b 20 b 8 b 5 b 10 b 10 b 10 b	LC-MS/MS	Schenck et al. (2019)
Lithuania	Grass	14	AFB_1	6	43	2−10	5.8	0.2	HPLC	Vaičiulienė et al. (2021)
Hungary	Legumes	2	OTA	2	100	2.52−27.57	15.05	0.5	HPLC	Adácsi et al. (2022)
Silage										
Netherlands	Grass	120	ZEN RC	7 1	6 1	NR−308 81	93 81	NR	LC-MS/MS	Driehuis et al. (2008)
Australia	Grass and legumes	16 11 11 11	ZEN AFB_1 ZEN DON	13 3 9 7	81 27 82 64	300−79,800 a 9−13 60−4,740 132−1758	16,020 a 10.7 1,143.3 737	100 a 1 32 50	HPLC	Reed and Moore (2009)
Lithuania	Grass and legumes	54	AFs ZEN DON	NR	NR	2.2−40 30−800 100−1100	48.6 291 413.8	2 10 100	ELISA	Baliukonienė et al. (2012)
Czech Republic and United Kingdom	Grass and legumes	12	AOH ENNs	NR	25 25	NR−148 a NR−571 a	16 a 20 a	NR	LC-MS/MS	Zachariasova et al. (2014)
Turkey	Legumes	14	AFs	ND	ND	–	–	0.046−0.059	HPLC	Sahin et al. (2016)
Ireland	Grass	290	ZEN MPA RC ENNs BEA	1 6 4 85 17	0.3 2 1 30 6	NR	NR	NR	LC-MS/MS	McElhinney et al. (2016)
Lithuania	Grass and legumes	20	AFs DON ZEN T-2/HT-2	20	100	0−100 a 135−520 a 16−625 a 59−178 a	29.95 a 364.9 a 258.05 a 111.3 a	NR	ELISA	Jovaišienė et al. (2017)
England	Grass	51	MMA for 13 metabolites	ND	–	–	–	0.2−10	LC-MS/MS	Cogan et al. (2017)
Poland	Grass	33	FB_1 ZEN DON NIV STG ENNs BEA	6 1 12 9 4 22 16	20 3 37 27 13 67 47	NR−10.4 NR−9.14 NR−528 NR−14,262 NR−22 NR−345 20.8	7.24 9.14 139 4,473 9.27 11.15 3.76	1.2 0.9 15 9 0.45 0.15 0.15	LC-MS/MS	Panasiuk et al. (2019)
Tunisia	Grass and legumes	9	HT-2 ENNs	1 1	11 11	NR	22.4 0.12	1.97 0.29−0.51	GC-MS/MS LC-MS/MS	Juan et al. (2020)
Lithuania	Grass	78	AFB_1 ZEN DON T-2	76 78 58 78	97 100 74 100	1.3−34.2 83−683 <LOD−2386 <LOD−445	9 376 438 104	1 17 200 50	ELISA	Venslovas et al. (2021)
Lithuania	Grass	20	AFB_1 ZEN DON T-2	6 6 12 16	30 30 60 80	2−7 130−330 100−500 39−260	5.5 286.67 321.25 154.17	0.2 3 20 1.4	HPLC	Vaičiulienė et al. (2021)
Spain	Grass and legumes	33	AFs FBs	1 3	3.0 9	0.91−2.21 379.4−910.58	1.56 603.61	0.05−0.1 30	HPLC LC-MS/MS	Rodríguez-Blanco et al. (2021)

Table 1 – contd.

1	2	3	4	5	6	7	8	9	10	11
Finland	Grass and legumes	87	AOH	2	2	493−736 a	614.5 a	NR	LC-MS/MS	Manni et al. (2022)
			BEA	67	77	3−8,854 a	349.7 a			
			ENNs	37	43	3−2,452 a	447.1 a			
			MPA	30	34	17−84,830 a	7,915.3 a			
			RC	48	55	6−60,962 a	6,705.6 a			
			ZEN	10	12	17−19,992 a	2,720 a			
Portugal	Grass	6	FB_1	1	17	383.10	383.10	NR	LC-MS/MS	González-Jartín et al. (2022)
			OTA	1	17	341.23	341.23			
			CTN	1	17	701.99	701.99			
			ENNs	4	67	6.57−15.42	10.75			
			BEA	4	67	5.67−8.87	7.97			
			RC	3	50	35.16−40.21	38.16			
			STG	1	17	46.58	46.58			
Hungary	Legumes	2	OTA	1	50	0.8	0.8	0.5	HPLC	Adácsi et al. (2022)
			DON	1	50	450	450	100		

a – expressed as µg/kg dry matter; b – expressed as µg/kg air-dry sample.

N: number of samples analyzed; LOD: limit of detection; MMA: multi-mycotoxin analysis; ND: not detected; NR: not reported; ELISA: enzyme-linked immunosorbent assay; GC-MS/MS: gas chromatography coupled to tandem mass spectrometry; HPLC: high performance liquid chromatography; LC-MS/MS: liquid chromatography coupled to tandem mass spectrometry; AFB_1: aflatoxin B_1; AFB_2: aflatoxin B_2; AFG_1: aflatoxin G_1; AFG_2: aflatoxin G_2; AOH: alternariol; BEA: beauvericin; CTN: citrinin; DON: deoxynivalenol; ENNs: enniatins; FBs: fumonisins; FB_1: fumonisin B_1; FB_2: fumonisin B_2; HT-2: HT-2 toxin; MPA: mycophenolic acid; NIV: nivalenol; OTA: ochratoxin A; RC: roquefortine-C; STG: sterygmatocystin; T-2: T-2 toxin; ZEN: zearalenone.

The highest mean concentrations of ZEN (16,020 µg/kg), DON (2,140 µg/kg) and AFs (29.95 µg/kg) were observed in samples from Australian silage, Canadian hay and Lithuanian silage, respectively. OTA was reported in only 4 studies, and the highest mean level was 341.23 µg/kg. Other mycotoxins reported in the studies included FB_1, FB_2, BEA, ENNs, alternariol (AOH), cyclopiazonic acid (CPA), citrinin (CTN), mycophenolic acid (MPA), roquefortine-C (RC), sterygmatocystin (STG) and other trichothecenes than DON, such as nivalenol (NIV), diacetoxyscirpenol (DAS), T-2 and HT-2 toxins.

ZEN was detected in North America, Europe, Oceania, and the Middle East, and the highest mean concentrations were observed in silage. In two articles (Manni et al., 2022; Reed and Moore, 2009), the mean levels of ZEN found in grass and legume forages were above the tolerance limit of 500 µg/kg determined by the European Union in feed for young animals and dairy cattle (European Commission, 2016). DON was the most frequently detected trichothecene in North America, Europe, and Oceania. Mean concentrations were low, except in a study of hay in Canada that showed mean levels of 2,140 µg/kg (Raymond et al., 2000), above the tolerance limit of the European Union for young animals, 2,000 µg/kg (European Commission, 2016). T-2 and HT-2 toxins were detected in North America, Europe, and Africa in grass and legume hay, haylage, and silage. Only one study that tested hay (Raymond et al., 2000) showed that mean levels were above the tolerance limit of 250 µg/kg adopted by the European Union for mixed feeds, except for cats (European Commission, 2013). NIV was detected in grass and legume hay, haylage, and silage samples from North America and Europe, with the highest mean concentration of 4,473 µg/kg in silage (Panasiuk et al., 2019). However, despite the overall high frequencies of trichothecenes described in the evaluated studies, their levels in conserved forages were low.

AFs were identified in Europe, Oceania, and the Middle East. In three studies (Baliukonienė et al., 2012; Tsiplakou et al., 2014; Vaičiulienė et al., 2021), the mean AF levels were above the tolerance limit of 20 µg/kg determined by the Food and Drug Administration (FDA) in feed for young and suckling animals (FDA, 2016). Moreover, four articles (Hashemi, 2016; Reed and Moore, 2009; Vaičiulienė et al., 2021; Venslovas et al., 2021) described mean levels of AFB_1 above the tolerance limit 5 µg/kg established by the European Union in mixed feeds for young and suckling animals (European Parliament, 2002). OTA was found only in European samples of grass and legume hay, haylage, and silage. Two studies reported mean concentrations of 341.23 µg/kg (González-Jartín et al., 2022) and 276.6 µg/kg (Tsiplakou et al., 2014), which were above the tolerance limit of 250 µg/kg for OTA in raw materials for animal feed (European Commission, 2016). Among emerging mycotoxins, ENNs and BEA were detected in 6 studies conducted in Europe (Zachariasova et al., 2014; McElhinney et al., 2016; Panasiuk et al., 2019; Schenck et al., 2019; González-Jartín et al., 2022; Manni et al., 2022) and one in Africa (Juan et al., 2020).

Discussion

In this review, a great variation in the LOD values of analytical methods for determination of mycotoxins was demonstrated. For example, the LOD for ZEN varied from 250 µg/kg using ELISA (Raymond et al., 2000) to 100 µg/kg by HPLC (Reed and Moore, 2009), and 0.9 µg/kg by LC-MS/MS-based methods (Panasiuk et

al., 2019). Thus, comparisons regarding the frequencies of mycotoxins in conserved forages in different studies should consider the variations in the number of positive samples according to the LOD reported in each study. Because immunochemical techniques, such as ELISA, have lower cost and higher practicality, they are most used for rapid routine and on-site detection (Anfossi et al., 2016). Chromatographic-based techniques (HPLC, GC-MS/MS, and LC-MS/MS) provide a more sensitive and selective determination of mycotoxins (Tsiplakou et al., 2014), which could explain their use in 75% of the studies evaluated in this review.

The most frequent mycotoxins found in the conserved forages were ZEN, DON, and AFs, with small variations in their occurrence levels in the three types of products surveyed (hay, haylage, and silage). The high incidences of mycotoxins reported indicate the occurrence of failures in production, handling or storage processes of conserved forages, as the conservation techniques aiming at moisture reduction, pH reduction, and anaerobiosis are designed precisely to prevent fungal growth (Andersen et al., 2020). In addition, some mycotoxigenic fungi including *A. fumigatus*, *P. roqueforti*, *P. paneum* and *F. oxysporum* can develop even under unfavorable conditions of low oxygen levels as provided by conservation techniques (Andersen et al., 2020; Buszewska-Forajta, 2020; González-Jartín et al., 2022).

ZEN is a mycoestrogen mainly produced by *F. graminearum* and *F. culmorum*. Pigs are the most sensitive species, but clinical signs have also been observed in humans and in cattle. In general, animals exposed to ZEN show endocrine dysregulation symptoms, such as infertility and reduced pregnancy rates, as well as immunosuppression (Logrieco et al., 2018; Mahato et al., 2021). Thus, the highest levels of ZEN reported in silage (Manni et al., 2022; Reed and Moore, 2009) could be considered as risk factors for food-producing animals, especially young animals. Although ZEN is not carcinogenic and shows relatively low toxicity, there is some evidence of a relationship between the presence of this mycotoxin and the occurrence of idiopathic precocious puberty in Italy, China, and the United States (Logrieco et al., 2018; Manni et al., 2022).

Trichothecenes are also mainly produced by fungi in the *Fusarium* genus, and are the largest mycotoxin group, with around 170 metabolites. However, only some of them are relevant for human and animal mycotoxicosis, the A-Type, T-2, HT-2 and DAS; and the B-Type, DON and NIV. In humans and animals, they affect proliferating cells, causing immunosuppression and inhibition of cell functions, leading to apoptosis, growth delay, necrosis, digestive syndromes, and neuroendocrine changes (Logrieco et al., 2018; Mahato et al., 2022). However, the mean concentrations of trichothecenes in conserved forages were low, hence indicating low risk for animals fed these types of products.

AFs are mainly produced by *A. flavus*, *A. parasiticus*, and *A. nomius*. They are found worldwide but are more common in tropical and subtropical climates. The most abundant and toxic form, AFB_1, is considered by the International Agency for Research on Cancer (2002) as group 1 carcinogen, given its relationship with the occurrence of hepatocellular carcinoma. In humans, AFs intoxication cause immunosuppression, mutagenicity, teratogenicity, and impaired child growth. In production animals, these toxins may cause weight gain and inadequate food conversion, besides liver disorders (Daou et al., 2022; Mostrom and Jacobsen, 2020). Aflatoxin M_1 (AFM_1) is the biotransformed form of AFB_1 excreted in milk and body fluids of animals. Although ten times less toxic than AFB_1, AFM_1 is also classified by the International Agency for Research on Cancer as group 2B carcinogen, a possible carcinogen to humans (Logrieco et al., 2018). AFM_1 is the only mycotoxin that is considered in milk regulations, with tolerance limits ranging from 0.05 µg/kg by the EU, to 0.5 µg/kg by the FDA (Zeidan et al., 2022). Around 0.5–6% AFB_1 is converted to AFM_1, and its presence in animal products is a public health hazard, especially for children and babies, since milk is a major constituent of their diets (Ceniti et al., 2021; Daou et al., 2022). In this review, four articles (Hashemi, 2016; Reed and Moore, 2009; Vaičiulienė et al., 2021; Venslovas et al., 2021) reported high mean concentrations of AFB_1 in conserved forages, stressing the need for prevention measures applied to forage preservation techniques to avoid animal health risks and the occurrence of AFM_1 in animal-derived foods.

OTA is produced by fungi in the genera *Aspergillus* and *Penicillium*, and *A. ochaceus* and *P. virridicatum* are the most commonly involves species. This mycotoxin inhibits protein synthesis and humoral factors, showing nephrotoxic and immunosuppressing activity in humans and animals, besides being classified as group 2B carcinogen by the International Agency for Research on Cancer (Logrieco et al., 2018; Tolosa et al., 2021). In addition, OTA is involved as a causative agent of the fatal Balkan endemic nephropathy (Tolosa et al., 2021). The occurrence of OTA is more frequent in regions of temperate climate (Logrieco et al., 2018). Coherently, OTA was reported only in conserved forages from European countries (González-Jartín et al., 2022; Tsiplakou et al., 2014). In some countries such as Denmark, Estonia, Romania, and Italy, there are tolerance limits for this mycotoxin in meat and edible viscera, such as kidneys and liver, ranging from 1 to 10 µg/kg (Tolosa et al., 2021).

The occurrence of BEA and ENNs was reported at high levels in conserved forages from Europe and Africa. These mycotoxins are generally produced by different species of *Fusarium*, and ENNs may also be produced by other fungal genera, such as *Alternaria* and *Verticillium*. Concerns related to emerging mycotoxins are similar to those involving other mycotoxins: deleterious effects on animal health and performance, and the risk to humans posed by their transfer to animal products (Křížová et al., 2021). Other mycotoxins were also found only in conserved forages from European countries, such as FBs,

RC, AOH, MPA, STG, CTN, but in lower frequencies and at low levels (Driehuis et al., 2008; González-Jartín et al., 2022; Manni et al., 2022; McElhinney et al., 2016; Panasiuk et al., 2019; Rodríguez-Blanco et al., 2021; Schenck et al., 2019; Zachariasova et al., 2014). In spite of the low frequency and concentrations, these mycotoxins need to be constantly investigated, especially FBs, for which there are regulations in different countries. FBs are normally classified as *Fusarium* toxins, as they are produced by several species in this genus. The main ones are *F. verticillioides* and *F. proliferatum*. However, other genera may also produce this toxin, such as *A. niger*. FBs interfere with cell growth, differentiation, and communication, leading to toxicity and carcinogenicity. Studies in animals show that these toxins may cause neural tube defects. FB_1, the most toxic metabolite, is classified as group 2B by the International Agency for Research on Cancer (Logrieco et al., 2018). In equines, it may cause leukoencephalomalacia, and in pigs, pulmonary edema (Mostrom and Jacobsen, 2020; Tolosa et al., 2021).

An important outcome from studies that used LC-MS/MS methods is the occurrence data regarding co-contamination of conserved forages with two or more mycotoxins in one given sample. It is well known that a single fungus species (e.g., from the genus *Fusarium* spp.) may produce more than one metabolite, while different fungi species may simultaneously contaminate the feed material and lead to the production of multiple mycotoxins (Lee and Ryu, 2017). Thus, the co-occurrence of mycotoxins in feedstuffs has been seen as an emerging feed safety problem, considering the possible interactions between them and their combined toxic effects (Palumbo et al., 2020). In this review, multiple mycotoxins in conserved forages were reported in more than 50% of the evaluated studies, mainly regarding the co-occurrence of *Fusarium* toxins with AFs, as well as ZEN with trichothecenes. Similar results were described in a systematic review on the occurrence of mycotoxins in fresh forages (pastures) by Aranega and Oliveira (2022), who observed that the main fungi metabolites found in this material were DON, ZEN, AFs, and FBs. This similarity between the mycotoxin occurrence in conserved and fresh forages, which are usually the material used for preservation, reinforces the need for strict control measures for preventing fungal contamination of forage plants before application of the preservation process, to avoid risk levels of mycotoxins in the conserved forages.

Conclusions

The mycotoxins most frequently found in grass and legume hay, haylage, and silage were ZEN, DON, and AFs. These mycotoxins, produced by species from the genera *Fusarium* and *Aspergillus*, were detected in conserved forages from North America, Europe, Oceania, and the Middle East. DON was reported at high frequencies, but at low levels in the evaluated studies, which may indicate low risk concerning the feed safety. However, the mean levels of ZEN, AFs and OTA reported in some studies were above the tolerance limits for animal feed determined by the EU and FDA, thus requiring stringent control measures to reduce the animal exposure to those mycotoxins through the diet. The fact that the co-occurrence of two or more mycotoxins was observed in 62% of the studies analyzed warrants concern about the high frequencies of fungi contamination in conserved forages.

Although data presented here paints the general picture on the occurrence of mycotoxins in grass and legume conserved forages, this issue should be more widely studied comparing different grass and legume forages, conservation techniques, detection methods, and the interaction between mycotoxins. More studies on the occurrence of mycotoxins in grass and legume conserved forages in Africa, America, Asia, Oceania, and the Middle East are necessary, given the importance of these feeds in animal production in these regions.

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Conflict of interest

The authors declare that there are no conflicts of interest relevant to this review.

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