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REVIEW ARTICLE

Fungi and mycotoxins in silage: an overview

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Summary

The present revision shows the early and current knowledge in the field of silage fungi and mycotoxins explaining the relevance of fungi and mycotoxins in silage. The problem does not end in animal disease or production losses as mycotoxins in feed can lead to the presence of their metabolic products in dairy products, which will be eventually affecting human health, mainly infants. Silage is green forage preserved by lactic fermentation under anaerobic conditions. This ecosystem maintains its quality and nutritional value depending on interactions among physical, chemical and biological agents. Forages used for ensilage are naturally in contact with yeasts and filamentous fungi, and the contamination often occurs in the field and can also occur during harvesting, transport, storage. Moreover, postharvest poor management can lead to a rapid spoilage. Studies on fungal contamination of dairy cattle feed have shown how corn silage influences the contamination degree of feed supplied to livestock. Increasing knowledge in this area will help elucidate the influence that this microbiota exerts on production and/or degradation of mycotoxins present in silage. Some of these fungi, although opportunist pathogens, are relevant epidemiologically and represent a high risk of contamination to farm workers who handle them improperly.

Introduction

Silage consists in green forage preserved by spontaneous lactic fermentation under anaerobic conditions (Miller 2001). The primary purpose of making silage is to maximize the preservation of original nutrients in the forage crop with minimum losses in the nutritional quality that allows its use as fodder during periods of feed scarcity, avoiding the seasonal nature of production and increasing the animal stocking per hectare.

The production of silage has a long history of tradition with evidences of plant materials preservation as animal feed in the ancient Egypt, 1500–1000 BC. Also, the Greeks and the Romans appreciated silage production and from ancient writings it can be derived that airtight sealing was seen as a precondition for successful silage preservation. In 1877, a French farmer named Goffart published the first book on ensilage, which was seen as the practical modernization of the procedure. By the 1900s, ensiling was a common, although not dominant, mean of preserving crops in both Europe and North America. Even though the basic principles of silage production had been applied for many centuries, the success story of silage was delayed until the 1950s, when intensive animal production was demanded. The revival of this preservation method was also supported by the introduction of the forage harvester in Europe and North America in the 1960s (Wilkinson and Toivonen 2003). At present, this practice is considered one of the most appropriate forms to preserve the nutritional value of animal food. Farmers

in countries like Holland, Belgium, Germany and Denmark, store more than 90% of their forage as silage.

Production of Silage

The two most common options for storing forages are ensiling and haymaking. The predominant storage in a region varies mainly by climate. Countries with predominantly dry climates, such as the United States and Australia, preserve most of their forage as hay (Table 1). In contrast, most northern European countries store forages as silage due to their wet climates (Hutnik and Kobielak 2012). Even in countries with good weather for haymaking, such as France and Italy, about half of the forage is ensiled to 100 million tons dry matter (Wilkinson and Toivonen 2003). Argentina produced 8·4% of world agricultural output and accounted for 2·9% of world agricultural trade over the period 2005–2007 (Lence 2010). Since the 1990s, the production has remained more or less static. Table 1 presents the silage production from some of the most important silage producer countries in the world.

The most important crops for ensilage are grass and maize, with grass being more important in Europe and maize in North America. Other crops for ensilage include corn varieties (e.g. wheat, barley), legumes and industrial by-products (sugar beet tops, pressed sugar beet pulp, brewer's grains) (Wilkinson and Toivonen 2003).

Corn and hay crops (legumes, grasses and legume–grass mixtures) are ensiled frequently in the Midwest. The chemical composition and therefore the fermentation characteristics of corn and hay crops differ greatly. Corn has low concentrations of protein and some minerals, but high concentrations of fermentable carbohydrates. Hay crops vary in protein but when harvested at the proper stage of maturity usually have a high concentration of protein and are good sources of minerals. Hay crops, however, have low concentrations of fermentable carbohydrates.

Table 1 Silage and hay production in selected countries in 2000 (adapted from Wilkinson and Toivonen 2003)

	Silage			
	Hay	Grass	Corn	Other
Country	(million Mg DM)			
Australia	4·5	0·9	0·3	0·04
Canada	45·0	na	2·8	4·8
Chile	0·6	1·3	na	na
France	22·5	3·1	16·8	5·3
Italy	15·1	0·2	6·9	0·4
Japan	1·5	2·2	1·1	0·07
New Zealand	0·4	0·6	0·3	0·02
Spain	3·1	1·7	0·7	0·2
United Kingdom	2·5	9·4	1·1	0·4
United States	138	1·7	32·4	9·0

na, Estimate not available; Million Mg DM, million megagram dry matter.

Hay making is difficult in many tropical regions because at the time when the forage is of acceptable quality for forage conservation to be worthwhile, which is normally early in the wet season, the weather is likely to be too unreliable for sun drying. Artificial drying is expensive and facilities are not widely available. Silage on the other hand can be made using fresh or, preferably, wilted material ('t Mannetje 1999).

Filamentous Fungi in Silage

Storage systems for feed are artificial ecosystems that maintain the quality and nutritional value depending on the interaction among physical, chemical and biological agents. Fungal spoilage and mycotoxin contamination are one of the greatest risks of stored feed like silage. Fungal growth leads to loss of nutrients and dry matter, palatability reduction, reduction in silage consumption, which generates as a consequence the losses in animal performance (O'brien *et al.* 2008). Forages used for making silage are naturally in contact with yeasts and filamentous fungi present in the field, but can also occur during harvesting, transport and storage; poor management at postharvest can lead to rapid spoilage. Critical water activity for safe storage is 0·7–0·8 (Magan and Aldred 2007). When this level is exceeded, the degrading fungi as *Eurotium* spp, *Penicillium* and *Aspergillus* species can grow, and the increase in respiratory activity produces the increase in temperature of the silage that can lead to the development of other fungi especially thermophilic ones causing further deterioration. Fungal concentrations higher than 1×10^4 CFU g^{-1} in forage may be the reason for respiratory problems, abnormal ruminal fermentation, decreased reproductive function, kidney damage, skin and eye irritation (Scudamore and Livesey 1998). Fungi that produce mycotoxins belong to two groups: those commonly called field fungi and those called storage fungi. Among toxicogenic field fungi are plant pathogens such as *Fusarium graminearum* (deoxynivalenol, nivalenol) that grow on senescent or stressed plants or *F. verticillioides* (fumonisins) and sometimes *Aspergillus flavus* (aflatoxins) and fungi that initially colonize the plant and predispose the commodity to mycotoxin contamination at storage such as *Penicillium verrucosum* (ochratoxins) and *A. flavus* (aflatoxins) (Adams 1977).

Table 2 shows the predominant fungal contamination in silage during 2000–2011 years. The distribution of fungal genera and species is quite similar in among different countries. The main fungi isolated were *Aspergillus*, *Penicillium* and *Fusarium* genera followed by *Mucor*,

Table 2 Fungal contamination in silages 2000–2011

Origin of sample	Type of silage	Isolated fungi	Reference
Argentine	Corn	*Aspergillus flavus, A. parasiticus, A. fumigatus, A. terreus, Penicillium sp, Fusarium sp*	Alonso *et al.* (2009)
Argentine	Corn	*A. flavus, A. parasiticus, A. fumigatus, A. terreus, Penicillium roqueforti, P. paneum, P. griseofulvum, P. crustosum, Fusarium solani, F. equiseti, F. verticilloides*	Gonzalez Pereyra *et al.* (2011)
Egypt	Corn	*A. flavus, A. fumigatus, A. niger, A. ochraceous, A. versicolor, F. oxysporum, Penicillium sp*	El-Shanawany *et al.* (2005)
France	Corn	*A. fumigatus, A. parasiticus, F. verticillioides, P. roqueforti, P. brevicompactum, P. purpurogenum*	Garon *et al.* (2006)
France	Corn	*A. fumigatus, Monascus ruber, P. roqueforti, A. flavus, A. parasiticus, A. fumigatus, Byssochlamys nivea, F. culmorum, F. graminearum, F. verticillioides*	Richard *et al.* (2007)
Iran	Corn	*A. flavus, A. fumigatus, A. niger, Fusarium, Penicillium, Cladosporium, Mucor, Yeast*	Khosravi *et al.* (2008)
Ireland	Grass	*P. roqueforti, P. paneum, Geotrichum sp, Fusarium sp*	O'brien *et al.* (2008)
Mexico	Corn	*Aspergillus sp, Fusarium sp, Penicillium sp, Mucor sp*	Reyes-Velázquez *et al.* (2008)
Portugal	Corn	*Aspergillus sp, A. fumigatus*	Dos Santos *et al.* (2002)
Slovakia	Corn	*Acremonium sp., A. flavus, Fusarium sp, Mycelia sterilia, Mucor circinelloides v. Tiegh., M. racemosus, Rhizopus stolonifer, Paecilomyces variotii, Penicillium sp., Scopulariopsis sp.*	Bíro *et al.* (2009)

Byssochlamys, *Absidia*, *Arthrinium*, *Geotrichum*, *Monascus*, *Scopulariopsis*, *Stachybotrys* and *Trichoderma*. A high incidence of potentially toxicogenic species such as *A. flavus*, *A. parasiticus*, *A. fumigatus*, *F. verticilloides*, *F. graminearum P. roqueforti*, among other species. Although previously *P. roqueforti* strains had been reclassified into three species (*P. roqueforti*, *P. paneum* and *P. carneum*), on the basis of molecular genetic and biochemical profiles. Gonzalez Pereyra *et al.* (2011) only distinguish *P. roqueforti* and *P. paneum* among other 67 species by morphological characterization. Moreover, O'brien *et al.* (2008) discriminated between *P. roqueforti* and *P. paneum* by morphological and molecular characterization. Recently, Van Asselt *et al.* (2012) informed a high incidence of *Fusarium* species and their mycotoxins as contaminants of corn silage in a survey in the Netherlands.

Reports from Argentina and Brazil informed fungal counts present in different sections of silos: the upper section – generally more exposed to fungal contamination, the middle section – the best-preserved portion and the lower section. They showed that contamination levels were significantly different at each ones (Gonzalez Pereyra *et al.* 2008, 2011; Alonso *et al.* 2011; Keller *et al.* 2013). The upper and lower sections had the same levels, whereas the middle section showed low fungal contamination. Proper storage is related to the state of compaction; the most compact the silo, the least possibility of losing reduced pH and anaerobic conditions. González Pereyra *et al.* (2008) and Keller *et al.* (2012) reported that physical properties in silages varied according to the sampled section. Some samples had a_W, temperature and pH levels able to allow fungal growth and mycotoxin production. The physical factor that assures the silage preservation is pH. Upper, low and border silo samples showed pH values over 6, suggesting probable fungal development and showing poor silage management.

Most of the studies published in the world have shown the incidence of fungi present in silages. However, few of them have detailed this contamination in pre- and postfermented silos. Gonzalez Pereyra *et al.* (2008) and Keller *et al.* (2012) reported fungal counts, fungal genera frequency and mycotoxins present in pre- and postfermented corn silos, whereas Keller *et al.* (2012) reported the same previous studies from pre- and postfermented sorghum silage. The fungal count is a technique used to estimate the hygienic quality of the substrate; in this sense, counts over 1×10^4 CFU g^{-1} are considered the limit recommended by the good manufacturing practices in animal feed (GMP 2008). *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp., the main mycotoxigenic fungi were the prevalent genera in corn silage samples in Argentina at counts most of the times over the recommended limit. In Brazil, the same substrate showed 25% of prefermented and 70% of postfermented samples exceeded the limit recommended as a quality standard for animal feeds. Total fungal counts increased during the fermentation process being higher in post- than in prefermentation samples. *Aspergillus flavus* and *A. fumigatus* relative abundance in postfermented silage samples was higher than that in prefermented samples. In contrast, *P. griseofulvum*, *P. funiculosum* and *F. graminearum* were isolated only in prefermented samples.

Recently, Gonzalez Pereyra *et al.* (2011) reported a comparative analysis of the mycobiota present in two kinds of silos: trench type and silo bags. Trench silos are often made over a concrete base or on the bare ground, often between concrete walls, where the fodder is placed in large heaps, rolled by a tractor to push air out and wrapped in plastic covers held by recycled tires. Silage may also be emptied into a bagger, which puts silage into a large plastic bag that is laid out on the ground. This type of silo is usually known as silo bag. Currently, the choice of the type of silo to be used only depends on costs. Thus, trench silos are economically attractive to store large amounts of feedstuff, but its design leads to an important loss of material because of the high exposure to the environment. When the silage is stored in bags, the influence of climate is reduced to a minimum and fermentation losses are very low, but the bag and the bagging process increase costs. In this study, mycobiota analysis revealed that yeasts, *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. prevailed in corn silage on both, trench silos and silo bags. The importance of the isolation of these genera from silage relies on the fact that the main mycotoxin producer species were present and *Aspergillus flavus* was the dominant species able to produce aflatoxin B_1 (AFB_1). *Penicillium* species included potential roquefortine C and confirmed patulin (PAT) producers. In trench silo samples, *F. verticillioides* was the dominant species among others, whereas in silo bag samples, *F. verticillioides* was the only species isolated. Airborne contaminants such as *Cladosporium* spp. and Mucorales that were isolated from trench silo samples were not present in silo bags, presumably because of the limited contact of the samples with the open air and soil.

Mycotoxins Contamination, Toxigenic Profile of Strains and Mycotoxicoses

Studying the pre-existing mycobiota in a given commodity can sometimes be used as a guideline to estimate the mycotoxins that could potentially be contaminating the substrate. However, mycotoxins are more resistant than mycelia to the feedstuffs processing and storing conditions as evidenced by their presence in samples where the mould can no longer be isolated. Animal feed is frequently contaminated simultaneously by several fungi, which are able to produce several toxins each. However, little is known about synergy among toxins present simultaneously in these substrates. Most silage is made from annual crops, and mycotoxin content may vary from year to year. In addition, distribution of mycotoxin contamination is very heterogeneous existing highly contaminated sites among good quality material.

Main mycotoxins found in silages include aflatoxins (AFs), particularly aflatoxin B_1 (AFB_1), besides ochratoxin A (OTA), fumonisins B (FBs), trichothecenes as deoxynivalenol (DON) and gliotoxin, among others. Table 3 shows the main mycotoxins found in silages. Corn silage is the most studied; however, there are few data on mycotoxin contamination in sorghum and grass silages. Moreover, scarce data about tropical silages have been reported. While conditions to promote growth and mycotoxin production are well known, reduced oxygen conditions and acidity present in the silage do not favour such processes.

Table 3 Mycotoxins occurrence in silage during 2000–2011

Origin of sample	Type of silage	Mycotoxin found	Analytical Tech	References
Argentina	Corn and Sorghum	AFs DON	ELISA	Amigot *et al.* (2006)
Argentina	Corn	AFs	HPLC	Alonso *et al.* (2009)
Argentina	Corn	AFB_1 ZEA DON FBs patulin	HPLC-	Gonzalez Pereyra *et al.* (2008, 2011)
Brazil	Corn and Sorghum	AFB_1	TLC	Sassahara *et al.* (2005)
Belgium	Corn	DON, FBs, ZEA Beauvericin	HPLC-MS-MS	Van Pamel *et al.* (2011)
Denmark	Corn	ZEA DON GLY fumiclavine A roquefortine A and C mycophenolic acid	HPLC-MS-MS	Rasmussen *et al.* (2010)
Egypt	Corn	AFs T2	TLC	El-Shanawany *et al.* (2005)
France	Corn	AFB_1 ZEA DON CIT	HPLC-MS-MS	Garon *et al.* (2006)
France	Corn	GLY DON CIT	HPLC-MS	Richard *et al.* (2007)
France	Corn	AFB_1 DON CIT	HPLC-MS-MS	Richard *et al.* (2008)
Germany	Corn	Trichothecenes ZEA	GC-MS HPLC	Schollenberger *et al.* (2006)
Italy	Corn	AFB_1	HPLC	Decastelli *et al.* (2007)
Mexico	Corn	AFB_1 OTA FBs DON ZEA	ELISA	Reyes-Velázquez *et al.* (2008)
the Netherlands	Corn and Grass	ZEA DON Roquefortine C mycophenolic acid	HPLC-MS-MS	Driehuis *et al.* (2008)
Slovakia	Corn	AFs DON FBs T2 ZEA OTA	ELISA	Bíro *et al.* (2009)
USA	Corn	FBs	HPLC	Kim *et al.* (2004)

Ensilage is a raw material that influences the degree of contamination of finished feed supplied to cattle. Alonso *et al.* (2011) developed a multivariate analysis biplot graphs that demonstrated the association between finished feed with fungal and mycotoxin contamination of raw materials. It was important to emphasize that silage was the ingredient with the higher count of aflatoxigenic fungi.

Exposure of cattle to mycotoxins is usually given through consumption of contaminated feed. *Aspergillus fumigatus*, *P. roqueforti* and *Byssochlamys nivea* are some of the toxigenic fungi with a high incidence in silages (El-Shanawany *et al.* 2005; Richard *et al.* 2007). *Penicillium roqueforti* is an acid-tolerant species that can develop even in environments with very low oxygen and high concentrations of CO_2 and has been detected as a predominant species in different kinds of silages (Auerbach *et al.* 1998; O'brien *et al.* 2008). Other species isolated at high frequency is *A. fumigatus*, whose risk lies in its ability to produce gliotoxin, fumigaclavine toxins A and C and fumitremorgin, and it is an opportunistic pathogen that could produce pulmonary and respiratory diseases in humans and animals (Dos Santos *et al.* 2002; Pereyra *et al.* 2008).

Pre- and postfermented corn and sorghum samples were also studied for mycotoxins contamination. González Pereyra *et al.* (2008) reported that prefermentation corn silage samples showed no AFs contamination, whereas 17% of the postfermented ones were contaminated with AFB_1. Moreover, 67% of them showed levels over the tolerated limits in cattle feed (20 μg kg^{-1}). Keller *et al.* (2012), in the same way as for the presence of fungi, determined the mycotoxins levels present in pre- and postfermented corn silage samples in association with silage sections. Aflatoxin B_1, OTA, DON and FB_1 contamination was detected in corn silage pre- as well as postfermentation and in every sampled section of the silos – upper, low, lateral and central. Aflatoxin B_2, G_1 and G_2 were not detected in any of the samples. Aflatoxin B_1 contamination frequency and levels were higher on postfermentation silage samples when compared with prefermented samples, suggesting that *Aspergillus* section *Flavi* and AFB_1 contamination was enhanced during storage. Fumonisin B_1 and DON levels increased during storage, being higher in postfermentation samples, whereas there was no significant difference between OTA levels in pre- and postfermentation samples.

Gonzalez Pereyra *et al.* (2011) studied the differences between trench type and silo bags to establish an additional selection criterion to reduce the exposure risks of mycotoxins for human and animal health as well as the associated economic losses. Silo bag samples showed a high AFB_1 contamination but only 8·6% of them had levels that exceeded the recommended limit for meat bovines. In trench silos, a low incidence of AFB_1 contamination was found (14%). Fumonisin B_1 levels in both, trench silos or silo bag samples, were under the detection limits of the method, and DON levels in silo bag samples were below the limit recommended by the EU (12 μg g^{-1}). Zearalenone was not detected in trench silos, nor was in silo bags as well as PAT. In this study, all samples from silo bags that were positive for DON also contained AFB_1. Similarly, in a previous study, 83·5% corn silage samples were co-contaminated with three mycotoxins: ZEA, DON and FB_1 (González Pereyra *et al.* 2008). These authors stated that comparing trench silos and silo bags, when the handling is adequate, the reduction in fungi and mycotoxin contamination in the silo bag is considerable. However, if the plastic cover breaks or the handling is not proper, an increase in total CFU g^{-1} – especially the dominant and toxigenic species – can accelerate the deterioration. The reduction in contamination frequency of *Fusarium* spp. – as well as other nontoxigenic environmental fungal species – was considered an advantage of using silo bags. Moreover, the reduction in the occurrence of different mycotoxins in silo bags could be considered. In trench silos, the material remotion by shovelling and not with silage cutting machine could be considered disadvantage because the compaction of the material can be reduced increasing the risk of mould contamination.

Few works have stressed the importance to characterize the toxigenic profile of fungal species isolated from silages due to its mycotoxigenic potential under inadequate environmental conditions. Gonzalez Pereyra *et al.* (2011) showed that 17% *A. flavus*, 100% *F. verticillioides* and 100% *P. griseofulvum* and *P. paneum* isolated from corn silage in Argentina were able to produce AFB_1, FB_1 and PAT, respectively. Keller *et al.* (2013) studied the toxigenic profile of aflatoxins and OTA producers and observed that 60% *A. flavus* produced AFB_1 and AFB_2; 75% *A. parasiticus* $AFB_1/AFB_2/AFG_1/AFG_2$ and 33% *A. niger* aggregate produced OTA. Other species within *Aspergillus* genus isolated with a high incidence in silage is *A. fumigatus*. This micro-organism has a great micotoxicologic importance for its ability to produce gliotoxin and other tremorgenic toxins. Pereyra *et al.* (2008) determined the toxigenic ability of *A. fumigatus* isolated from corn silage and obtained 21·4% strains able to produce gliotoxin, fumitremorgin B, fumitremorgin B and fumigaclavine C, whereas only 7% were able to produce fumigaclavine C. In other work, Keller *et al.* (2012) determined the ability of *A. fumigatus* strains to produce gliotoxin and observed that the percentage of gliotoxin-producing strains were higher in postfermented samples compared with prefermented ones. Moreover, this result was observed in both corn and sorghum silage samples.

In cattle, chronic ingestion of mycotoxin causes several adverse effects, increased susceptibility to disease, loss of

reproductive performance and in case of dairy cattle, a decrease in yield and quality of milk production. These effects are caused because exposure of animals to mycotoxins causes a decrease in consumption or rejection of feed by animals, reducing the absorption of nutrients and impaired metabolism, alterations in the endocrine system and suppression of the immune system (CAST 2003). The biological effects of mycotoxins depend on the ingested amounts, number of occurring mycotoxins, and time of exposure and animal sensitivity. Moreover, the mycotoxin effects are not only amplified by stress production but also high in intensively reared cattle destined to meat or milk production (Yiannikouris and Jouany 2002; Binder 2006).

Conclusions and Public Health Significance

Cattle feed has a high proportion of silage. Although this substrate has been used for over 30 years, few information is available related to fungi and mycotoxin contamination. The high fungal counts present in silage suggest a high fungal activity that could affect the palatability of feed and reduce the animal nutrients absorption. For dairy cattle, the problem does not end in animal disease or production losses as the mycotoxins in feed can lead to their presence or their metabolic products in dairy products, which will be eventually affecting human health, mainly infants. The mycotoxin of major concern in cows' milk is aflatoxin M_1 (AFM_1), a metabolite produced by the biotransformation of AFB_1, which is detected in milk as a direct result of contaminated feed intake (Van Egmond 1989). The demonstrated toxic and carcinogenic effects of AFM_1 have led the International Agency for Research on Cancer-World Health Organization (IARC-WHO) to reconsider its carcinogenicity classification and to change it from group 2 to group 1 (IARC 1993). The potential presence of AFM_1 in milk and its by-products represents a worldwide concern because such products are mainly consumed by children, who are more susceptible to the adverse effects of mycotoxins (Boudra *et al.* 2007). This fact is very important as AFM_1 is resistant to heating processes such as pasteurization (Galvano *et al.* 1996). Another concern is that some of the fungi present in silos, although opportunist pathogens, are relevant epidemiologically such as *A. fumigatus* and represent a high risk of contamination to farm workers who handle them improperly. Increasing knowledge in this area will help elucidate the influence that this microbiota exerts on production and/or degradation of mycotoxins present in silage.

The information collected by the mentioned researchers enabled estimate the mycotoxicological risk of different ensiling practices (trench type and silo bag) and determined the most adequate method to minimize economic losses and reduce the hazard to animal and human health.

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