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Mycotoxin Contaminating Maize and Grass Silages for Dairy Cattle Feeding: Current State and Challenges

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Abstract

Silages may represent 50-80 % of the total amount of diet for dairy cows during the winter. There is overwhelming evidence of contamination of maize and grass silages with fungi and associated trichothecenes, fumonisins, zearalenone, aflatoxins, ochratoxin A as well as some emerging mycotoxins (agroclavine, andrastin A, beauvericin, enniatins, festuclavine, fusaric acid, mevinolin, mycophenolic acid, roquefortins, T2/HT2 toxins). From year to year, multi contaminations often occurred with seasonal variations. The levels and incidences of deoxynivalenol and zearalenone raise concerns about animal health, as the levels overpassed the EC regulatory limits (2006/576/EC) for deoxynivalenol in 2006-samples from Poland and for zearalenone in 2000-2001 maize silages from Germany. AFB1 levels exceeded the maximum acceptable level in samples from Argentina. Atypical high occurring mycotoxins such as roquefortine C (up to 36,000 µg/kg), mevinolin (up to 65,400 µg/kg) and mycophenolic acid (up to 35,000 µg/kg) were not covered by routine analysis and legislation. These mycotoxins can diversely affect feed intake, productivity, reproductive performance and animal health but acute intoxications causing death are rare. Monitoring of mycotoxin in silages is extremely necessary. Both European and international legislation concerning the maximum tolerable limits must be strict and should also be adapted to include the interaction impacts between toxins. Further studies are needed to evaluate mycotoxin attributed to carry-over via meat, edible offal and lactation. Reliable multi-detection methods must be developed and should cover the normalized sampling plan to cover the heterogeneous distribution within the silo, the seasonal variation and the requirement to detect low levels of these contaminants. Conjugated (masked) forms of the mycotoxins and the long-term impacts to the exposure to low contamination deserved further assessment challenges.

Keywords: Feed, silage, maize, grass, safety, mycotoxins, risk exposure.

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Introduction

Silages constitute major components of ruminant feed rations worldwide (Oldenburg and Höppner, 2003; Auerbach, 2006; Garon *et al.*, 2006; Driehuis *et al.*, 2008a). Different forages such as whole corn plant, wilted grass, corn cob mix or sugarbeet pulp can be preserved by ensiling. Due to the ease mechanisation from harvest to feed-out, the prolonged preservation of the feed and its use in different seasons, whole maize crop constitutes a major part of the material for cattle feeding in several parts around the world (Driehuis *et al.*, 2008a, Richard *et al.*, 2009). Ensiling of immature cereals (wheat or barley) has recently received increasing interest due to the low prices of these cereals. In order to ensure good performance and animal health, it is of great importance to produce silages of high energy and nutrients contents and of good hygienic quality. During storage, silages are at risk from infection by a number of different fungi, ubiquitous in the nature (Weinberg and Muck, 1996). The moulds activity in silages results in heat production and dry matter (DM) losses which lower the nutritional value of the silage. Fungal development can cause discoloration, textural and flavour changes of the forage that are often associated with a decrease in the palatability and feed intake by animals. Moreover, some moulds may produce mycotoxins which are hazardous to the health of animals. Disease outbreaks due to the consumption of contaminated food and feedstuffs are a recurring problem worldwide. Co-occurrence of several mycotoxins in the commodities could reveal new hazards due to synergistic or additive effects; suggesting a revision of the limits of maximal acceptable levels either in feed or in food (Völkel *et al.*, 2011). In addition, the most difficult challenges were the presence of hidden or masked mycotoxins (Tran and Smith, 2011) or the impact of low concentration of these mycotoxins on meat quality. Certain mycotoxin can be transmitted from livestock diets to animal products such as milk (where aflatoxin M1 is concerned), edible offal, meat and therefore pose a food safety hazard (Fink-Gremmels, 2008; Bryden, 2012; Sumantri *et al.*, 2012). With respect to these challenges, this review focuses on the occurrence of various types of fungi and associated mycotoxins in silages, made of

maize and grass materials. It is devoted to be informative not only for breeders but also for consumers and scientists in this field to pave the way for future research dealing with the chemical risk related to mycotoxin contaminated feedstuffs and the remediation strategy regarding food safety.

Background

Brief Description of the Ensiling Process

Ensiling is a forage preservation method based on spontaneous lactic acid fermentation under anaerobic conditions that lowers the pH to a level at which clostridia and most molds growths are inhibited (Weinberg and Ashbell, 2003). The primary goal of making silage is to maximize the preservation of original nutrients in the forage crop for feeding at a later date. Ensiling also aims at preserving forage from undesirable micro-organisms spoilage. Once the fresh material has been stacked and covered to exclude air, the ensiling process can be divided into 4 stages (Weinberg and Muck, 1996).

Phase 1 - Aerobic Phase

In this phase, atmospheric oxygen is still present between the plant particles, and the pH is 6.0-6.5. These conditions enable the continuation of plant respiration and the activity of enzymes, such as proteases and carbohydrases, aerobic and facultative aerobic microorganisms, such as fungi, yeasts and enterobacteria.

Phase 2 - Fermentation Phase

Once air is removed, due to the plant and microorganisms respiration, the fermentation can begin (normally after a few hours after ensiling). The epiphytic lactic acid bacteria (LAB) ferment the water-soluble carbohydrates (WSC) from the crop to lactic acid and to a lesser extent to acetic acid. Due to the production of these acids, the pH of the ensiled material decreases to 3.8-5.0 and spoilage micro-organisms are inhibited. This fermentation continues for several days or weeks, depending on the properties of the ensiled forage crop and the ensiling conditions. If the fermentation proceeds successfully, LAB populations develop and become the predominant population.

Phase 3 - Stable Phase

As long as air is prevented from entering into the silo or container, relatively few changes occur. Most micro-organisms of phase 2 slowly decrease in numbers. Some acid-tolerant microorganisms survive this period in an almost inactive state. Others, such as clostridia and bacilli, survive as spores. Only some acid-tolerant proteases and

carbohydrases and some specialized microorganisms, such as *Lactobacillus buchneri*, continue to be active at a low level. Amounts of common fermentation end products in various silages are given in Table 1.

Table 1: Amount of common fermentation end products in various silages.

Item	Alfalfa silage		Grass silage	Corn silage	
	30-35 % DM	45-55 % DM	25-35 % DM	35-40 % DM	70-73 % DM
pH	4.3-4.5	4.7-5.0	4.3	3.7-4.2	4.0-4.5
Lactic acid (%)	7-8	2-4	6-10	4-7	0.5-2.0
Acetic acid (%)	2-3	0.5-2.0	1-3	1-3	<0.5
Propionic acid (%)	<0.5	<0.1	<0.1	<0.1	<0.1
Butyric acid (%)	<0.5	0	<0.5	0	0
Ethanol (%)	0.5-1.0	0.5	0.5-1.0	1-3	0.2-2.0
Ammonia-N (% of CP)	10-15	<12	8-12	5-7	<10

DM = Dry Matter

Phase 4 - Feed-Out Phase or Aerobic Spoilage Phase

This phase starts as soon as the silage is exposed to air. During feed-out, this is unavoidable, but it can start earlier due to damage to the silage covering (e.g. by rodents or birds). The process of spoilage can be divided into two steps. The primary spoilage stage is the onset of deterioration due to the degradation of preserving organic acids by yeasts and, occasionally, by acetic acid bacteria. This will cause a rise in pH, thus allowing microorganisms (bacilli, moulds, enterobacteria) that were inhibited by low pH to grow and further spoil the mass. This second spoilage stage is also associated with increasing temperature. Aerobic

spoilage occurs in almost all silages that are opened and exposed to air. However, the rate of spoilage is highly dependent on the numbers and activity of the spoilage organisms in the silage. Spoilage losses of 1.5-4.5 % DM loss per day can be observed in affected areas. These losses are in the same range as losses that can occur in airtight silos during several months of storage.

In order to produce silages of good hygienic quality with minimal nutrient losses it is important to control and optimize each phase of the ensiling process (Weinberg and Muck, 1996; Weinberg and Ashbell, 2003). Many factors can affect what type of fermentation takes place in a silo (Figure 1).

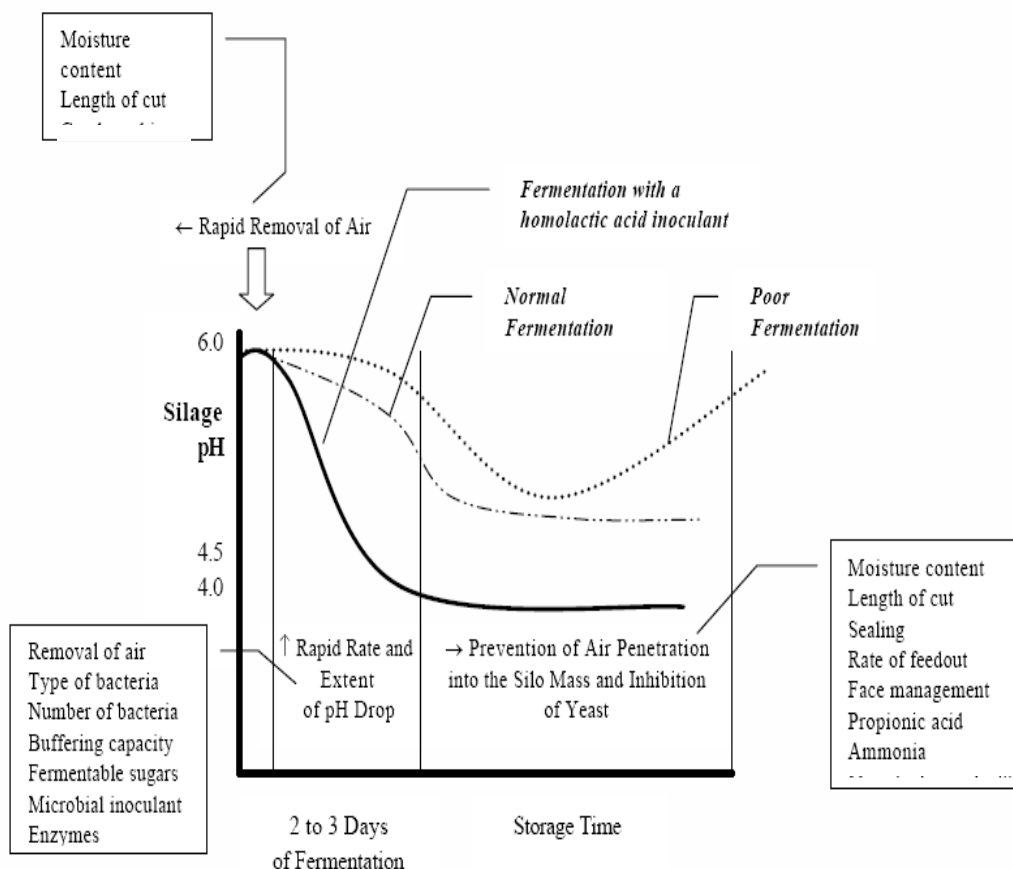


Fig. 1: The three major events that make good silage and factors that can affect the silage fermentation process (Kung, 2000).

For example, delayed sealing which prolongs the aerobic phase may result in reduction in the WSC available for fermentation and in the lactic acid produced in the silage (Weinberg and Muck, 1996). DM content of the forage can also have major effects on the ensiling process via a number of different mechanisms (Weinberg and Ashbell, 2003). First, drier silages do not pack well and thus it is difficult to exclude all of the air from the forage mass during the confection of the silo. Second, as the DM content increases, growth of lactic acid bacteria is curtailed and the rate and extent of fermentation is reduced. Third, if the forage is too moist and pH decline is not sufficient, clostridia, which ferment lactic to butyric acid and amino acids to ammonia, might become active. This process is referred to as “secondary or clostridia fermentation”, and it results in increases in pH and losses of silage DM content. The type and numbers of microorganisms (LAB, clostridia or moulds) on

the plant also have profound effects on silage fermentation.

Significance of Silages in Animal Feedings in Some European Countries

The popularity of silages in modern farming systems has increased because of the low production costs and high nutritional value (Wilkinson and Toivonen, 2003). In Germany, silage production is the biggest on-farm preservation operation. In 2000, a total of 76.2 million tons of silage were produced compared to only 2.3 million tons of hay (based on fresh matter). Maize silage represents the major proportion (about 58%), followed by grass and legume silage (about 32%) and whole-crop cereal silage (about 2%) (Auerbach, 2006). In addition, harvested fresh forage maize is conserved by ensiling before being fed to ruminants (Oldenburg and Höppner, 2003). In the Netherlands, maize silage may constitute 50–75% of the daily diet for a dairy cow consuming

~25 kg/day of DM (Eastridge, 2006, Driehuis *et al.*, 2008b). In Belgium, preservation of forage crop by ensiling is commonly used to ensure a continuous and consistent supply in dairy husbandry throughout the year. Silages generally represent 50-80 % of the diet of dairy cows during the winter. It is possible to ensile almost any plant material, including plant by-products like brewery grains and sugar beet pulp. The main forage crops in Belgium are grass (282,886 ha in 2010), fed fresh or as silages or hay, and maize, generally fed as whole crop silage (176,314 ha cultivated in 2010). Maize grains are also preserved by ensiling process as Corn Cob Mix (CCM) or as hard drought grain, for feeding beef cattle and pigs. With 1,124 and 804 ha (in 2010), the significance of leguminous silages made of pea and bean, respectively, is quite minor (INS, 2012).

The Danish maize silage production is enough to feed ~410,000 dairy cows eating silage all year around, if the maize plants are harvested at the recommended 30% DM content (Jensen *et al.*, 2005). Maize for green fodder covered 6% (159,000 ha) of the Danish cultivated area in 2008 and in total 6.26 million tons of maize silages were produced representing a value of approximately 200 million EUR. During the summer, approx. 50% of the Danish dairy cows are turned to pasture (Rasmussen, 2010), whereas maize and grass silage mixed with concentrates are the most common feeds during the winter time. Baled grass silage accounts for approximately 0.3 % of silage fed to livestock in Ireland (O'Kiely *et al.*, 1998). Typically bales are harvested between May and September and stored for 5–10 months before feeding to livestock from November to April (O'Brien *et al.*, 2005).

Filamentous Fungi Contaminations in Silage

The occurrence of moulds has attracted considerable attention as potential causes for poor performance and health disorders in domestic livestock. Studying the dynamics in the microbiology of maize silage during the whole season storage, Storm *et al.*, (2010) have found that the risk of fungal spoilage was highest during 5-7 months after ensiling and lowest after 11 months. Seasonal variations were also observed with some species like *Aspergillus fumigatus* which

Harvested forage crops, such as maize and grass, harbor a diverse mycobiota consisting of pathogenic, commensal and endophytic fungi that are associated with the growing plant. Additionally, forage can be contaminated during harvesting and chopping, by propagules of saprotrophic fungi from air, soil and ambient dust. Whereas a normal ensiling process eliminates most undesirable microorganisms from the field (Mansfield and Kulda, 2007), sometimes silages can be a pool of deleterious bacteria, such as *Bacillus cereus*, *Clostridium tyrobutyricum*, *Clostridium botulinum*, *Escherichia coli* O157 and *Listeria monocytogenes* (Fenlon and Wilson, 2000; Thylin, 2000) and also several deleterious filamentous fungi.

Visible fungal growth on grass and maize silages in farms are common and moulds usually develop in the surface layers of the silage, where oxygen is present (Driehuis *et al.*, 2008a,b). Growth conditions vary according to the mould and under poor storage conditions the whole silage can become mouldy, particularly in silage exposed to air ingress, condensation, heating, leakage of rainwater, insect infection and/or managerial variation (Muck, 1988). *Fusarium* and *Alternaria* are the main pre-harvest field fungi (Rasmussen, 2010) that are able to survive the fermentation process under acidic and anoxic conditions (Samson *et al.*, 2002). As for post-harvest fungi, genera such as *Aspergillus*, *Byssosclamyces*, *Geotrichum*, *Paecilomyces*, *Penicillium*, *Monascus*, *Mucor* and *Trichoderma* are also able to tolerate organic acids, carbon dioxide and the low availability of oxygen and may be detected in silage. *Aspergillus fumigatus* is harmful occurred in silage (Storm *et al.*, 2010) together with a high diversity of *Penicillium* such as *P. roqueforti* and *P. paneum*, species which are acid tolerant and can grow at low levels of oxygen and high levels of CO₂ (Auerbach *et al.*, 1998; Storm *et al.*, 2010). significantly decreased between October-December and February-April in samples from Normandy in France (Lanier *et al.*, 2010). The presence of fungi causes a reduction in feed value and palatability of the silage (DiCostanzo *et al.*, 1995). Table 2 presents some important mould species that have been regularly isolated from silages.

Trends of Mycotoxin Contamination in Silages

Panel of accompanied mycotoxins in silages is presented in Table 3.

Major and Emerging Mycotoxins in Silages

Very often, silages were contaminated via the raw material obtained in the field with mycotoxin produced by *Fusarium* species such as deoxynivalenol (DON), zearalenone (ZEN), (T2/HT2 toxins (T2/HT2), ENNs, nivalenol (NIV), Fumonisin (FB), Fusaric acid (FA), beauvericin (BEA)) and less routinely determined *Alternaria* toxins (*Alternariol* (AOH), *Alternariol monomethyl ether* (AME)) (Figure 2A). Note that maize silage was a major source of DON and ZEN intakes which often co-occurred in this commodity (Signorini *et al.*, 2012). Co-occurrence of mycotoxins is widespread in these studies and the majority of tested samples were contaminated with more than one mycotoxin. Concentrate feed and corn silage may provide up to 56% and 51% of the total DON and ZEN taken from the diets, respectively.

According to the EC recommendation (EC, 2006), the maximum acceptable DON contents in feed materials such as maize by-products was estimated at 12,000 µg/kg. Note that Grajewski *et al.*, (2012) have found DON levels overpassing this

level in 2006 samples from Poland (Table 3; Figure 3). ZEN loads also exceeded the acceptable limit of 3,000 µg/kg set by 2006/576/EC in 2000-2001 maize silages from Germany (Oldenburg and Höppner, 2003). As for FBs, the maximum tolerable content in maize by-products was 50,000 µg/kg for FB1+FB2 (2006/576/EC). None of the sample of silages included in this review reached this tolerable level. The mono-acetylated DON derivative 15AcDON often co-occurred with DON together with ZEN at ratio of 0.4-1.1 (Driehuis *et al.*, 2008). AcetylDON (3AcDON) was also frequently found in combination with DON together with 15AcDON. These derivatives and other xenobiotics can be partly metabolized by animals consuming silages and they are therefore not just exposed to the native mycotoxins, but also to their derivatives or hidden or masked mycotoxins formed by the plants. Still just little is known about the occurrence, bioavailability and further metabolism of these compounds, which additionally also escape established analytical detection techniques (Berthiller *et al.*, 2007). Reliable detection of these compounds in silages may be hindered by the requirement to detect low levels.

Table 2: Important moulds, potential mycotoxins in silages and associated toxic effects.

Fungal species (major sources)	Mycotoxins	Effects
Pre-harvest infestation		
<i>Alternaria spp</i>	Alternariol, alternariol monomethyl ether, tenuazonic acid, altenuene	Antifungal, phytotoxic, cytotoxic, fetotoxic, teratogenic, mutagenic, genotoxic
<i>Fusarium avenaceum</i>	Beauvericin	DNA damage, cardiotoxicity
<i>F. graminearum</i> , <i>F. culmorum</i>	Deoxynivalenol	Feed refusal, diarrhea, emesis, reproductive failure
<i>F. avenaceum</i> , <i>F. acuminatum</i> , <i>F. compactum</i>	Enniatins	Cytotoxicity in mammalian cells
<i>F. verticillioides</i> , <i>F. proliferatum</i>	Fumonisin	Hepatotoxic, nephrotoxic
<i>F. venenatum</i> , <i>F. poae</i> , <i>F. equiseti</i> , <i>F. sambucinum</i>	T-2 toxins	Immunosuppressive reproductive failure, haemorrhagic
<i>F. graminearum</i> , <i>F. culmorum</i> , <i>F. equiseti</i>	Zearelanone	Oestrogenic
Post-harvest infestation		
<i>Aspergillus flavus</i> , <i>A. parasiticus</i>	Aflatoxins	Carcinogenic, hepatotoxic, DNA damage
<i>Penicillium sp.</i>	Andrastin A, B, C	Inhibitors of antitumor agents
<i>P. citrinum</i> , <i>P. verrucosum</i> , <i>A. terreus</i> , <i>Monascus ruber</i>	Citrinin	Nephrotoxic, embryotoxic
<i>A. versicolor</i> , <i>A. cyclopium</i>	Cyclopiazonic acid	Vasodilator, Neurotoxic endoplasmic reticulum inhibitor
<i>A. fumigatus</i>	Fumigaclavine A, C, fumitremorgen	Haemorrhaging, diarrhoea, tremorgenic
<i>A. fumigatus</i>	Gliotoxin	Hematuria, antiviral, antifungal, antibacterial immunosuppressive
<i>P. brevicompactum</i> , <i>P. roqueforti</i> , <i>P. carneum</i> , <i>Byssosclamyces nivea</i>	Mycophenolic acid	Immunosuppressive, antiviral, antifungal, antibacterial, antitumor
<i>Monascus ruber</i> , <i>A. terreus</i>	Mevinolins (Monacolins)	Hypocholesterolemic
<i>A. ochraceus</i> , <i>P. verrucosum</i>	Ochratoxin A	Carcinogenic, nephrotoxic, teratogenic, immunosuppressive
<i>P. expansum</i> , <i>B. nivea</i> , <i>Paecilomyces variotii</i> , <i>P. paneum</i>	Patulin	Carcinogenic, pulmonary oedema, haemorrhaging
<i>P. roqueforti</i>	PR toxin	Liver and kidney damage, mutagenic
<i>P. roqueforti</i> , <i>P. carneum</i> , <i>P. crustosum</i> , <i>P. expansum</i> , <i>P. griseofulvum</i>	Roquefortine A, B, C	Neurotoxic
<i>A. versicolor</i> , <i>A. nidulans</i>	Sterigmatocystin	Hepatotoxic, carcinogenic, teratogenic
<i>A. fumigatus</i>	Verruculogen	Tremorgenic

Sources: Scudamore and Livesey, 1998; Frisvad *et al.*, 2006; Hollmann *et al.*, 2008; Föllmann *et al.*, 2009.

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Table 3: Mycotoxin contamination of silages from recent surveys.

Countries (years)	Type of Silages	Mycotoxins	Incidence (%) / positive (n*)	Average (µg/kg)	Maximum (µg/kg)	References
<i>Pre-harvest mycotoxins</i>						
Argentina (2005-2006)	Maize	ZEN	-	50	350	Gonzalez Pereyra <i>et al.</i> , 2007
(2005-2006)	Cereals (not specify)	DON	-	276	870	Roige <i>et al.</i> , 2009
		FB1	-	1,110	2,490	
		DON	34% (38)	-	1,000	Signorini <i>et al.</i> , 2012
		ZEN	16% (38)	-	1,560	
		T-2	18% (38)	-	-	
(2001-2003)		DON	35% (191)	-	-	
		ZEN	82% (121)	-	-	
Austria (2002-2003)	Whole plant maize	DON	7 (?)	1,086	2,332	Lang <i>et al.</i> , 2005
	Crop maize		4 (?)	3,555	5,550	
			14 (?)	311	798	
	Whole plant maize	ZEN	7 (?)	39	69	
	Crop maize		14 (?)	25	79	
Belgium (2009-2010)	Maize Cutting surface Behind (1 m)	DON	4 (5) 5 (5)	124 107	262 140	Van Pamel, 2011
	Cutting surface Behind (1 m)	FA	1 (5) 2 (5)	33 61	45 180	
	Cutting surface Behind (1 m)	ENN B	2 (5) 5 (5)	11 20	12 25	
	Cutting surface Behind (1 m)	ENN B1	3 (5) 2 (5)	16 12	37 18	
Denmark (2006)	-	ENN B	95% (20)	73	218	Sørensen <i>et al.</i> , 2008
		ENN B1	40% (20)	10	48	Rasmussen <i>et al.</i> , 2010
		BEA	25 % (20)	8	63	
(2007– 2009)	Maize (hot spots)	DON	2 (10)	990	1,092	
		NIV	2 (10)	140	138	
	Maize (hot spots)	ZEN	2 (10)	71	156	
	Maize (unspoiled)		4 (10)	99	311	

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	parts) Maize (hot spots)	ENN B	3(10)	93	200	
		AOH	1 (10)	236	236	
		AME	1 (10)	51	51	
Egypt	Not specify	T2-toxin	3 (40)	-	-	El-Shanawany <i>et al.</i> , 2005
France (2003-2004)	Corn	DON	10 (11) 10 (11)	1,823 1,943	- 3,914	Lanier <i>et al.</i> , 2010
Germany (2000-2001)	Maize	ZEN	61% (56)	270	3,260	Oldenburg and Höppner, 2003
	Maize	DON	100% (5)	2,919	3,944	Schollenberger <i>et al.</i> , 2006
		NIV	100% (5)	1,612	2,809	
		15AcetylDON	100% (5)	59	127	
		HT2	100% (5)	18	26	
		SCIRP	20% (5)	124	124	
		MAS	60% (5)	30	49	
		ZEN	100% (5)	432	1,790	
The Netherlands (2002-2004)	Maize	DON	72% (140)	651	3,142	Driehuis <i>et al.</i> , 2008a
	Wheat		10 % (30)	62	1,165	
	Maize	ZEN	49% (140)	92	943	
	Grass		6% (120)	17	308	
	Maize	FB1	1.4% (140)	199	26,200	
	Maize	FB2	0.7% (140)	56	7,800	
Poland 2006	Not specify	DON	49/51	241	2,141	Grajewski <i>et al.</i> , 2012
2007			50/50	532	5,651	
2008			46/54	609	14,470	
2009			31/35	331	1,140	
2007		NIV	18/19	89	359	
2008			9/10	125	526	
2009			27/35	80	241	
2007		HT2-toxin	17/18	23	110	
2008			15/21	33	149	
2009			29/35	16	53	
2006		ZEN	16/41	12	116	
2007			49/50	16	415	
2008			44/53	43	1,150	
2009			34/35	29	129	
2007		FB1, FB2, FB3	19/19	2,260	6,821	
2008			2/3	15	18	
2009			13/16	30	82	
Serbia	Corn	ZEN	2 (18)	500	500	Skrinjar <i>et al.</i> , 2011
Switzerland (2005- 2006)	Maize	DON	100% (19)	1,350	2,990	Eckard <i>et al.</i> , 2011
		NIV	42% (19)	521	760	
		AcDON	21% (19)	218	300	
		HT2	26% (19)	95	130	
		T2	42% (19)	36	84	
		ZEN	79% (19)	181	430	
USA (2001-	Maize (Corn)	FB1	97% (89)	590	1,824	Kim <i>et al.</i> , 2004
		FB2	72 % (89)	66	276	

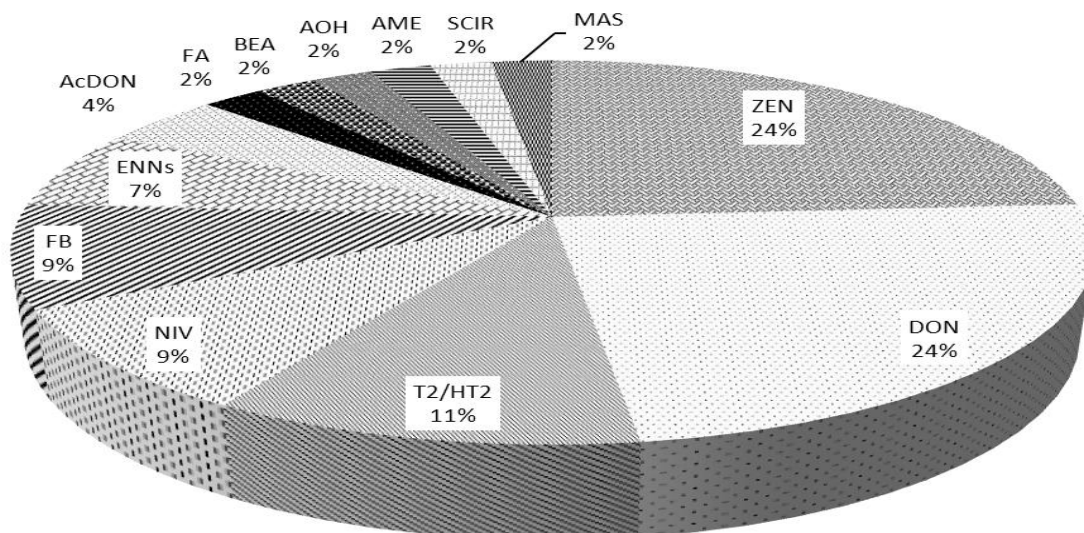
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2002)		FB3	57% (89)	29	174	
<i>Post-harvest mycotoxins</i>						
Argentina	Maize (Corn)	AFB1	17% (38)	-	156	Gonzalez Pereyra <i>et al.</i> , 2007
(2001-2003)		AFB1	73% (277)	-	-	Signorini <i>et al.</i> , 2012
Belgium (2006-2007)	Maize Grass	ROC	81 % (42) 57% (40)	1,200 600	15,100 7,200	Tangni <i>et al.</i> , 2010
(2009-2010)	Maize Cutting surface Behind (1 m)	ROC	4 (5) 2 (5)	18 34	52 90	Van Pamel <i>et al.</i> , 2011
Denmark	Corn (hot spots)	MPA	6 (10)	507	1,646	Rasmussen <i>et al.</i> , 2010
		ROC	3 (10)	11,826	33,662	
		GTX	2 (10)	594	906	
	Maize (hot spots)	AND A	6 (10)	2,400	8,811	
	Maize (unspoiled parts)		7 (10)	159	691	
Egypt	-	AF B ₁ + B ₂	9 (40)	-	-	El-Shanawany <i>et al.</i> , 2005
		AF	1 (40)	-	-	
		B ₁ +B ₂ +G ₁ +G ₂	2 (40)	-	-	
Germany (1997-1998)	Maize Grass	MPA	28 % (135) 37% (98)	690 2,200	23,000 35,000	Schneweis <i>et al.</i> , 2000
(2000-2001)	Grass Maize	MEV	23 (98)		65,400	Schneweis <i>et al.</i> , 2001
		STE	2 (40)	1,280	5,910	Oldenburg and
			69% (64) 86% (50)	1,440 1,100	12,890 10,870	Höppner, 2003
(1993)	Maize Grass	ROC	100% (12) 75% (12)	17,000 (DM) 6,000 (DM)	36,000 (DM) 15,000 (DM)	Auerbach <i>et al.</i> , 1998
Ireland (2003-2004)	Grass	AND	5 (5)	-	20,000	O'Brien <i>et al.</i> , 2006
		MPA	5 (5)	-	20,000	
		ROA	2 (5)	-	5,000	
		ROB	2 (5)	-	1,000	
		ROC	2 (5)	-	20,000	
		ROD	2 (5)	-	1,000	
		FC	2 (5)	-	1,000	
		MA	3 (5)	-	1,000	
		AC	2 (5)	-	1,000	
Netherlands (2002-2004)	Corn (core) Corn (surface)	ROC	25% (16) 50% (16) 100% (7)	96 1,605 25,986	- - -	Driehuis <i>et al.</i> , 2008b

				Corn	13% (16)	100	-	
				(Moulded	19% (16)	128	-	
				spots)	44% (16)	568	-	
				Grass (Core)				
				Grass				
				(Surface))				
				Mixture				
			MPA	Corn (core)	0% (16)	<25	-	
				Corn	50% (16)	660	-	
				(surface)	71% (7)	9,311	-	
				Corn	0% (16)	<25	-	
				(Moulded	13% (16)	40	-	
				spots)	38% (16)	256	-	
				Grass (Core)				
				Grass				
				(Surface)				
				Mixture				
Serbia	-	Corn	OTA	8 (18)	21	86		Skrinjar <i>et al.</i> , 2011
Poland		Not specify	OTA	42/50	1	1		Grajewski <i>et al.</i> , 2012
2006				3/20	1	19		
2007				3/11	1	11		
2008				0/6	nd	-		
2009								
USA		Maize	PAT	23% (120)	80	1,210		Mansfield <i>et al.</i> , 2008
		(Corn)						
			MPA	42% (120)	160	1,300		
(2001 –								
2002)			CPA	37% (120)	120	1,430		
			ROC	60% (120)	380	5,710		

n = Total analyzed samples; (-): missed data; DM = Dry matter; AC = Agroclavine; AF = Aflatoxin; AME = Alternariol monomethyl ether; AND = Andrastin; AOH = Alternariol; BEA = Beauvericin; CIT = Citrinin, CPA = Cyclopiazonic acid; DON= Deoxynivalenol; ENN = Enniatin; FA = Fusaric acid; FB = Fumonisin B; FC Festuclavine; GTX = Gliotoxin; MA = marcfortine A; MAS = Monoacteylsclirpenol; MEV = Mevinolin (Monacolin); MPA = Mycophenolic acid; NIV = Nivalenol; OTA = ochratoxin A; PAT = Patulin; ROA (B, C, D) = Roquefortin A (B, C, D); SCIRP = Scirpentriol; ZEN= Zearalenone.

MYCOTOXIN CONTAMINATING MAIZE AND GRASS SILAGES ...



B

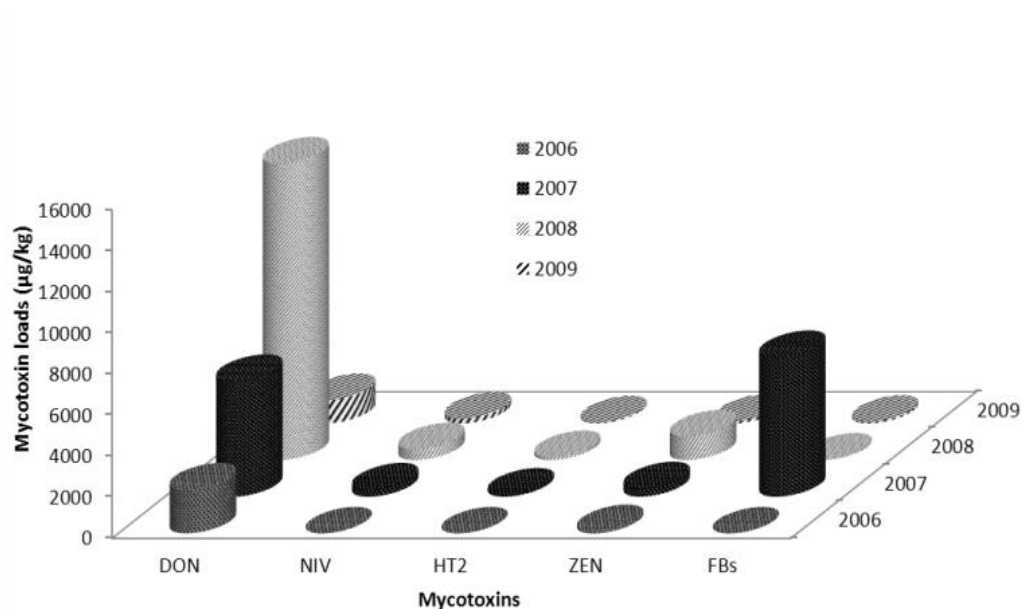
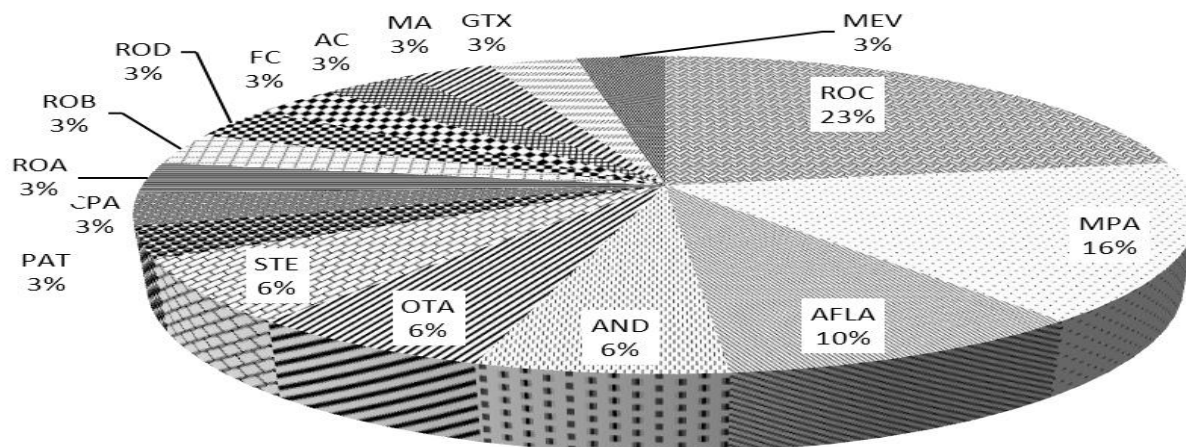


Fig. 2: Prevalence of pre-harvest (A) mycotoxin identified in different studies (n =16) performed during last decades and maximum DON concentrations as observed in Poland corn silages (B) during 2006-2009 as observed by Grajewski *et al.*, (2012). AcDON = Acetyl DON, AME = Alternariol monomethyl ether; BEA= Beauvericine; DON = Deoxynivalenol; ENN = Enniatin; FA = Fusaric acid; FB = Fumonisin B; FC Fesclavine; MAS = Monoacteylsirpenol, NIV= Nivalenol; SCIRP = Scirpentriol; T2/HT2 = T2/HT2 toxin (These graphs are made from data compiled in this review).

Under various environmental factors during postharvest and storage, *P. roqueforti* and *P. paneum* mainly were developed during ensiling and may produce ROC, MPA along with andrastin A (AND), roquefortines A, B and D (ROA, ROB, ROD), festuclavine (FC), marcfortine A (MA) and agroclavine (AC) (O'Brien *et al.*, 2005, 2006, 2007; Storm *et al.*, 2010). OTA, gliotoxin (GTX) and MEV produced by *Aspergillus*, *Monascus* and

OTA content in silage did not exceed the 250 µg/kg, set as maximum acceptable limit (EC, 2006). The evidence that AFB1 in silages may exceed the maximum level recommended (20 µg/kg) for animal welfare (EC, 2006) occurred in samples originated from Argentina (Gonzalez-Pereyra *et al.*, 2007).

A

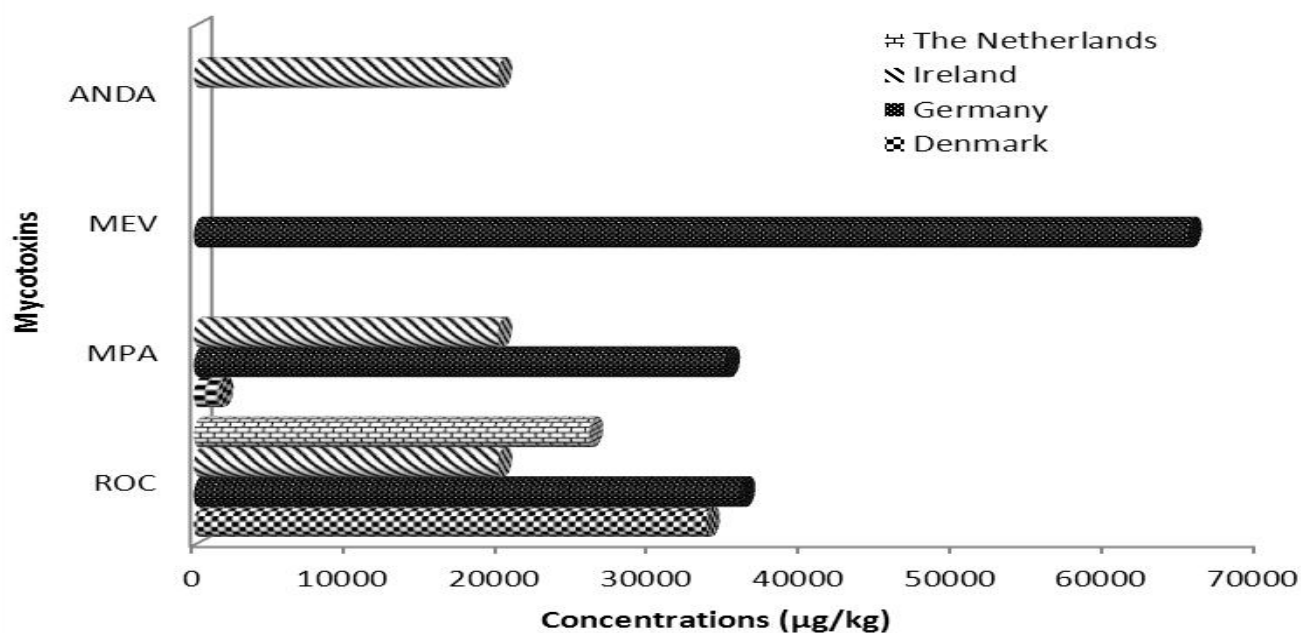


Mycoraceae species also occurred (Figure 3A).

Fig. 3: Frequencies of post-harvest (A) and levels (B) of mycotoxin identified in different studies (n = 16) performed during last years (ROA, ROB, ROC and ROD = roquefortine A, B, C and D, MPA = mycophenolic acid, AND = andrastin, FC = festuclavine, MA = marcfortine A, AC = agroclavine, OTA = ochratoxin A, GTX = Gliotoxin PAT = Patulin CPA = Cyclopiazonic acid; MEV = Mevinolin (These graphs are made from data compiled in this review).

Remarkably, the most highly occurring mycotoxins such as ROC, MPA (Figure 3B) were not covered by routine analysis and legislation.

Maximum concentrations of ROC observed in Denmark (33,662 µg/kg, Rassmussen *et al.*, 2010), Germany (36,000 µg/kg, Auerbach *et al.*, 1998),



Ireland (20,000 µg/kg, O'Brien *et al.*, 2006), in the Netherlands (26,000 µg/kg, Driehuis *et al.*, 2008b) and of MPA in Germany (35,000 µg/kg; Schneewis *et al.*, 2001), Ireland (20,000 µg/kg O'Brien *et al.*, 2006) deserve further health concerns. Little information is available about the possible chronic effects of such high loads of mycotoxins as well as the possible interaction with other toxins in cattle.

Note that ROC, MPA as well as PAT and CPA were found in freshly harvested material; contradicting the belief that *Penicillium* toxin formation occurs exclusively during storage (Mansfield *et al.*, 2008). Yiannicouris and Jouany (2002) have indeed concluded that there is an uncertainty concerning the conditions under which mycotoxins are formed in silages.

Some authors reported about the capacity of *Mucorales* to produce ergot alkaloid, agroclavine and fumigaclovine or 3-nitropropionic acid that may cause an acute encephalopathy (Hollmann *et al.*, 2008). It was also reported the ability of *Penicillium* species to produce andrastin A, roquefortines A, B, and D, festuclavine, marcfortine A, agroclavine (O'Brien *et al.*, 2006), patulin (Tapia *et al.*, 2005), PR-toxin (Driehuis *et al.*, 2008), and of *A. fumigatus* to produce fumitremorgen B, C and gliotoxin (Santos *et al.*, 2002). Recently, citrinin, gliotoxin have been detected in mature corn silages (Richard *et al.*, 2007). These toxins may contribute to the increase of the susceptibility of animal to viral and bacterial infections and the exposure of dairy cattle to the multi mycotoxin contamination. We therefore suggest in profoundly investigating these mycotoxins in silages and other feedstuffs.

Within a stack or bale there are many ecological niches with variable mycotoxin loads (Figure 4). Driehuis *et al.*, (2008b) have found that extremely high concentrations of ROC (up to 26,000 µg/kg) and MPA (up to 9,000 µg/kg) were detected in visibly moulded areas in surface layers of corn silages collected in the Netherlands. These levels turned to be 96 and <25 µg/kg in the core samples, respectively. Similarly, Richard *et al.*, (2007) have concluded that mycotoxin contents were stratified within the silo (i.e. top to bottom). This means that the absence of visible mould does not guarantee the silage is free from mycotoxins and heavily infested silage does not necessarily contain high levels of mycotoxins. This finding may be explained by the fact that non-mouldy fractions are also contaminated, probably due to the size of mycelium which was not yet larger enough to allow its visual appearance, or due to the diffusion properties between the substrate and the mycotoxin allowing the transfer from mouldy side to non-mouldy counterpart before sampling or to the presence of competitors inhibiting the moulds before sampling. Reliable detection of mycotoxins in silages may be hindered by the heterogeneous distribution which requests a good sampling plan. It is recommended to add to a quantitative exposure assessment an uncertainty analysis.

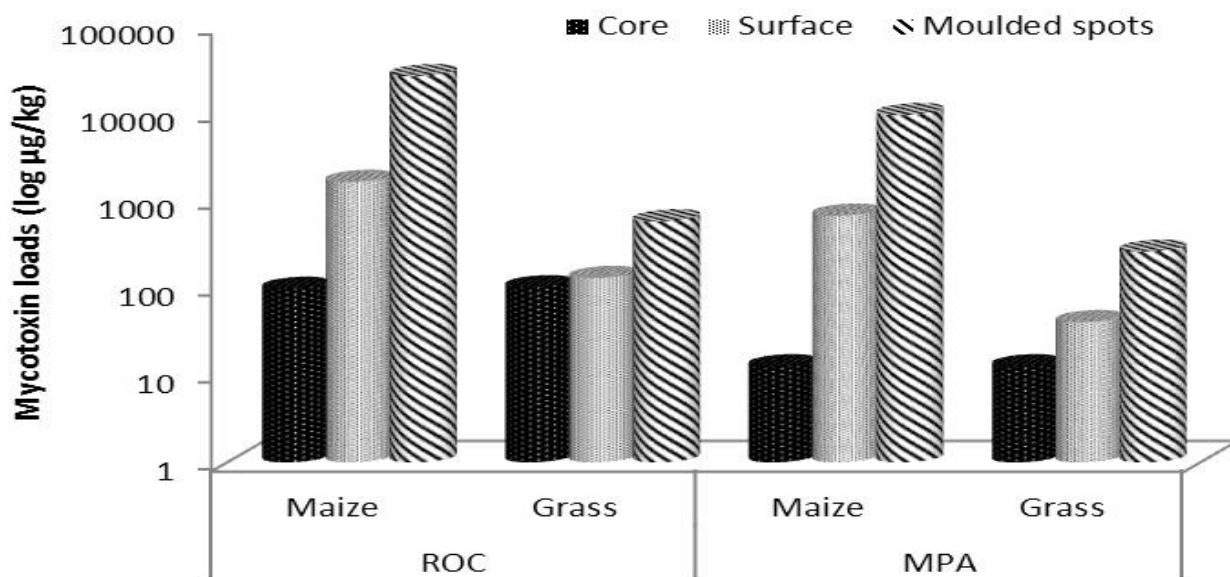


Fig. 4: ROC and MPA loads in different fractions of maize and grass silages from The Netherlands as observed by Driehuis *et al.*, (2008b) (This graph is made from data compiled in this review).

Regional Breakdown and Seasonal Variations in Mycotoxin Occurrences

Studies revealed certain similarities as well as differences in the distribution of contamination, often dependent on the geographical locations and meteorological conditions. AFB1 contamination of corn is usually associated with a tropical or subtropical climate (El-shanawany *et al.*, 2005; Gonzalez Pereyra *et al.*, 2007) and may not be a concern in Europe where DON, ZEN, ENNs, FBs, ROC, MPA and OTA are frequent contaminants of corn or grass silages (Driehuis *et al.*, 2008a,b; Grajewski *et al.*, 2012). However, it is noteworthy that climate changes may break this barrier and may lead to the occurrence of exotic fungi with unusual mycotoxin in European samples, as aflatoxin B1 was recently found in maize intended for animal feeding in Belgium. Exposure differs from year to year (Figure 2B) and therefore collection of additional information from year to year on the mycotoxin loads in silages from various countries should be encouraged in order to protect animal and human health. Therefore, more user-friendly and cheaper accurate analytical methods for detection of mycotoxins have to be developed as recently tested by Hiraoka *et al.*, (2013) for rice and maize silages.

Implication on Animal Health and Productivity

Risk of mycotoxicoses in animal farms is quite difficult to assess. Diagnosis of a mycotoxin-related disease is difficult because of nonspecific symptoms and wide ranging, interactions with other stress factors and difficulties in feed sampling and analysis. However, mycotoxins should be considered as a causative factor when unidentified problems exist. Depending on the type, amounts of toxins present in silage and the duration of exposure to mycotoxins, health problems on animal can range from minor digestive upsets, small fertility problems, reduced immune function and more rarely, to serious liver or kidney damage, abortions (Table 2) or animal death (Scudamore and Livesey, 1998; Yiannicouris and Jouany, 2002). The most

potent trichothecenes produced in silages are DON and T-2 toxin, able to be haematotoxic and immunosuppressive. An intestinal toxicity was observed for DON and its acetylated derivative, with a high toxicity of 15-AcDON when compared to the DON or 3-ACDON. The mycotoxin alteration of intestinal barrier function may increase bacterial translocation across the intestine and thus may enhance the susceptibility to enteric diseases. Moreover, the alteration of intestinal barrier by these mycotoxins may increase the intestinal passage of other xenobiotics including other mycotoxin (Oswald, 2013). They may also negatively affect rumen microorganisms (Rassmussen, 2010). A case investigation of dairy cattle fed mouldy corn containing 1 mg/kg T-2 toxin resulted in hemorrhagic syndrome. In animals, vomiting, feed refusal and diarrhoea are typical symptoms (Frisvad *et al.*, 2006). Mycotoxins can be primary agent causing acute health troubles in dairy herd that are specific to each toxin. More likely, they contribute to chronic problems with clinical symptoms that can be confused with other pathologies or cause an impaired rumen function (Fink-Gremmels, 2008). Aflatoxins have also been shown to negatively affect production, immune system function and rumen metabolism in cattle (Zain, 2011). The most common and most difficult challenges to identify mycotoxin occur when rations contain low levels of mycotoxins and the health effects are subclinical. Long term effects (i.e. carcinogenicity, endocrine disruption) as well as trans-generational toxicity of low amount of mycotoxins deserved further assessment challenges (Fink-Gremmels, 2013).

Carry-over of Mycotoxin to Animal Edible Products

Rumen microbiota is able to metabolize some mycotoxins such as DON to less toxic compounds. Lang *et al.*, (2005) showed that up to 10 mg DON were transformed completely within 24 hours into the significantly less toxic substance de-epoxyDON Metabolite (DOM-1). However, certain mycotoxins can be transmitted from livestock diets to edible animal products, such as milk, meat or edible offal and therefore pose a food safety hazard (Scudamore and Livesey, 1998; Yiannicouris and Jouany, 2002).

Signorini *et al.*, (2012) have successfully derived a positive correlation between mycotoxin loads in corn silages and the level of mycotoxin in dairy milk. They have found that concentrated feeds and corn silage were two ingredients highly correlated with the level of mycotoxin in dairy milk. The AFB1 proportion from these two ingredients was on average 57.1% of the total AFB1 taken from the diet. The ingestion of mycotoxin AFB1 by dairy cattle leads to the biotransformation of that substance, which is eliminated via milk as AFM1, and also causes damage to human health, especially for babies and children (Yiannikouris and Jouany, 2002; Seglar, 2003; Sassahara *et al.*, 2005). Up to 6% of the administered dose of aflatoxin is excreted in milk (EFSA, 2004).

Patulin is metabolized in the liver. Elimination pathways include faeces and urine. A carry-over of 2-3% in soft tissue and blood in general is possible (Völkel *et al.*, 2011). The carry-over rates from feed

to milk of *P. paneum* and *P. roqueforti* toxins (e.g. PR-toxin, roquefortines or festuclavine) are not known (Azziz-Baumgartner *et al.*, 2005). The occurrence of mycotoxins in milk (Table 4) makes it a particular risk for humans because of their negative effects in foodstuffs for adults and especially children (Prandini *et al.*, 2009). Moreover, the most prominent toxicological effects of the major classes of mycotoxins are recognized, but little is known about possible synergistic or antagonistic effects of mycotoxins and whether frequent exposure to low doses leads to chronic health problems (Driehuis *et al.*, 2008). No data on the carry-over rates of ROC and MPA from feed into milk are available (Driehuis *et al.*, 2008). According to Miller (2008), nothing is apparently known about the fate and distribution of ROC, PR-toxin or FC in milk, a question that needs resolution and holds special care owing to the importance of milk in infant and children diets.

Table 4: Products of ruminal bioconversion and transfer of mycotoxins from feed to milk.

Mycotoxins	Dose	Exposure duration (days)	Forms excreted	Concentration (ppb)
AFB1	350 µg/kg bw	3	AFM	0.10
DON	1,800 µg/kg bw	1	DON	<4
	66 mg/kg diet	5	DOM-1	30
	800 mg/kg diet	3	DOM-1 conjugate	220
FB1	3,000 µg/kg	14	FB1	0
OTA	50 mg (oral dose)	4	OTα	150
	1 g (oral dose)	4	OTA	100
			OTα	700
T-2 toxin	50 mg/kg diet	15	T-2 toxin	10-160
ZEN	25 mg/kg diet	7	ZEN	481
			α-ZOL	508
			β-ZOL	370
	40 mg/kg diet	21	ZEN	2.5
			α-Zearalenol	3.0

DON= Deoxynivalenol; DOM-1 = de-epoxyDON Metabolite FB1 = Fumonisin B1; OTA = ochratoxin A; OTα = ochratoxin α ZEN= Zearalenone, α-ZOL = α-Zearalenol

Other metabolites may be implicated in the toxicity of the parent molecule. Indeed, low OTA content may coexist with other derivatives (OTB, OTalpha (glucuronide or sulfate form), OTbeta, open-ring OTA, 4-hydroxylated OTA, 10-hydroxylated OTA, OTQ (Quinone form) and OTHQ (hydroxyquinone)) in liver or kidney and

may play a role in OTA toxicity (Pfohl-Leszkowicz *et al.*, 2012). We therefore recommend examining the levels of these metabolites that may increase the OTA toxicity and similarly for other molecules.

Mycotoxin Reduction Opportunities

Compared to other animals, ruminants are reported to be more resistant to mycotoxins than monogastric to many mycotoxins (Yiannicouris and Jouany, 2002; EFSA, 2005) partly due to inactivation or biotransformation by the rumen microorganisms (Bata and Lasztity, 1999; Halasz *et al.*, 2009). Mycotoxins can be partially detoxified by the activity of silage fermentative bacteria such as *Lactobacillus* and *Leuconostoc* species which biotransformed ZEN into α -ZEL, but not FB1 nor FB2. Note that mycotoxins can be metabolized to even more potent compounds (i.e. α -ZEL) in the rumen. Moreover, most strains were capable to bind these toxins (Boudra and Morgavi, 2008). High concentration of HT-2 found in silage proves that metabolism of T-2 toxin takes place during the fermentation process of ensiling. It is likely that the acute toxicity of both compounds is similar (Grajewski *et al.*, 2012). If some of the toxins or their metabolites may be fixed or bio-converted in animal tissue, most are eliminated via the urine, faeces and milk (Sumantri *et al.*, 2012). The complex microbial ecosystem of silage can also account for degradation and binding of mycotoxins.

The conditions of harvest, storage and feed production derived from corn and grass should be carefully controlled in order to reduce the exposure of dairy cattle to mycotoxins and subsequently reduce their carry-over in animal products. Preventive agricultural measures may be used in minimizing the risk of fungal and mycotoxin contamination (Signorini *et al.*, 2012). Agronomic measures play a key role in the control of mould growth and production of toxic metabolites on growing forage crops and therefore can significantly affect the amount of field-produced toxins in silage, especially in the context of climate changes. Due to genetic differences in susceptibility to fungal colonization, careful selection of crop varieties and use of sound, fungus-free seed form the basis of producing plant material of low mycotoxin contamination. The incidence of various moulds and their mycotoxins on growing crops can be markedly reduced by implementing balanced rotation systems and by choosing proper soil management practices. In addition, any injury to plant tissues caused by birds, machinery and insects should be minimized (Binder, 2007). Feedstuffs that not comply with the maximum levels set out by the

safety authorities should not be used as ingredient and mixed with other acceptable feed components which comply with the maximum levels.

Conclusions and Outlook

Information on the incidences and levels of mycotoxins in maize and grass silages showed their worldwide occurrence and highlights the presence of some emerging mycotoxins (enniatins, beauvericin, roquefortines, mycophenolic acid, mevinolin) besides the usual or classical mycotoxins (deoxynivalenol, zearalenone, ochratoxin A, aflatoxins, fumonisins). This review revealed that related compounds (acetylated or conjugated derivatives) may increase the bioavailability of mycotoxin in the tracts and merit to be detected and taken into account in animal or human risk assessment. Therefore, improvements of analytical methods developments for such mycotoxin screening and quantification are recommended. The challenges for the current generation and food authorities relate to setting appropriate regulatory limits that may improve animal and human health. Other feedstuffs such as concentrates, cotton seed, alfalfa hay, sugar beet pulp, hay, malt spent grains, sunflower cake or other agricultural feedstuffs can be a source of diverse mixture of mycotoxins that contaminate individual feed components and deserve further mycotoxin investigation. As many mycotoxins are unavoidable and can impair the health and productivity of dairy animals, we advise dairymen and their veterinarians to employ often a mycotoxin deactivator. Creating awareness among animal breeders, consumers, scientists for developing new methods for detection, exposure assessment and inactivation is of great importance for food safety.

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