F. Driehuis (2013) 22: 16–34

Silage and the safety and quality of dairy foods: a review

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Silage contains a number of potential hazards to the safety and quality of milk and dairy products. This paper reviews the present knowledge about silage as a source of (1) spores of anaerobic spore-formers (*Clostridium* species) and aerobic spore-formers (mainly *Bacillus* and *Paenibacillus* species), (2) the zoonotic pathogenic bacteria *Listeria monocytogenes* and *Escherichia coli*, and (3) mycotoxins. A distinction is made between field-derived mycotoxins, i.e. mycotoxins that are formed during growth of crops in the field, and ensilage-derived mycotoxins, i.e. mycotoxins that are formed after ensiling. The routes of transmission of these hazards from feed to milk, the effect of pasteurization of milk, and reduction strategies are discussed. Aerobic deterioration of silages is a major factor influencing levels of spores of both aerobic and anaerobic spore-formers, *L. monocytogenes*, and certain mycotoxins.

Key words: bacterial spores, milk quality, mycotoxins, pathogens, silage quality

Introduction

Food producers are responsible for the safety and quality of their products for consumers (European Commission 2002, European Commission 2005). Quality assurance of food products requires an integrated approach that assures safety and quality at all stages of the production chain. The safety and quality of milk and dairy products depend on the quality of raw milk produced at dairy farms, the quality of any other ingredients, processing conditions, and distribution and storage conditions. As suppliers of raw milk, dairy farms have an important role in the dairy production chain. Therefore, milk production at dairy farms needs to meet the demands and criteria with respect to animal health, feed quality and milking hygiene. The objective of dairy farm quality assurance is to prevent contamination of raw milk by residues of veterinary medicines and agricultural chemicals, environmental contaminants from for instance feed or soil and by harmful micro-organisms arising from feed, the housing system or the animals themselves. Feed is an important source of chemical and microbiological contaminants of milk (McEvoy 2002, Vissers and Driehuis 2009). The diet of high-yielding dairy cattle consists of two main classes of feedstuffs: forages and concentrates. Fresh, dried or ensiled forages generally constitute the largest fraction of the diet, usually 50 to 75%. Forage preserved as silage is the most popular form of forage in many countries. For example, grass and maize silage represented on average 67% of dry matter dietary intake of Dutch dairy cows in autumn and winter 2005 (Driehuis et al. 2008b).

This paper summarizes the present scientific knowledge about silage as a source of microbiological and chemical contaminants in the dairy chain. The paper focuses on three groups of safety or quality hazards of milk and dairy products: (1) spores of endospore-forming bacteria, such as *Clostridium* and *Bacillus* species, (2) the zoonotic pathogenic bacteria *Listeria monocytogenes* and *Escherichia coli*, and (3) silage-associated mycotoxins.

Spores of endospore-forming bacteria

Contamination pathway from silage to raw milk

Endospore-forming bacteria are an important group of contaminants of raw milk because of the resistance of the spores to heat and other adverse environmental conditions. Spores of many species survive pasteurization of milk and some even survive sterilization conditions. Important sources of bacterial spores are soil, silage and bedding materials. The main contamination pathway of spores from these sources to milk is shown in Figure 1. The original source of spores occurring in silage is often soil. Contamination of a crop by soil occurs during growth in the field or during harvesting. In crops that are ensiled, this soil contamination usually determines the initial spore concentration (Vissers et al. 2006, Vissers et al. 2007b). Whether a spore population will increase in concentration during ensilage depends on the properties of the micro-organism and the conditions prevailing in the silage. This will be discussed further on in this paper. Spores occurring in silage or other feeds that are consumed by a cow pass the gastrointestinal tract of the animal unaffected and are excreted with the faeces. There is evidence that spore concentrations increase during passage through the intestinal tract (Ali-Yrkkö and Antila 1975, Vissers et al.

Manuscript received July 2012

2007b), which can be explained by digestion of feed components. Vissers et al. (2007b) measured concentrations of spores of butyric acid bacteria in mixed grass and maize silage, faeces, bedding materials and raw milk from 24 Dutch dairy farms and observed that the concentration in faeces was on average about three times higher than the concentration in silage. Bedding materials that are used in barns where cows are housed usually become contaminated by excreted faeces. Under normal dairy farming practices, for instance when cows are lying in the barn, it is inevitable that bedding and faeces attach to the surface of the cow's udder and teats.

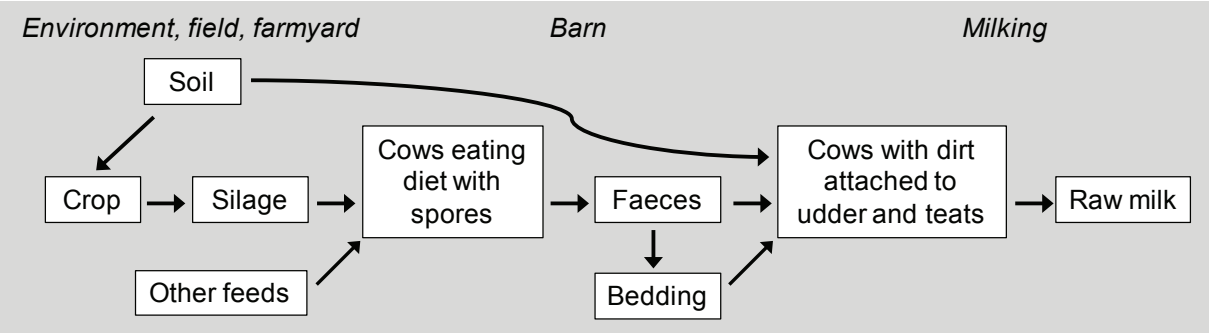

Fig. 1. Contamination pathway of bacterial spores from silage and other feeds to raw milk.

Good dairy farming practice requires that teats are cleaned before milking (FAO and IDF 2011). However, since teat-cleaning methods are relatively inefficient from a microbiological perspective, a fraction of bacteria and spores from dirt and faecal matter remains attached to the teat surfaces and is rinsed off during milking operations. An evaluation of different manual teat-cleaning methods revealed that spore concentrations in milk were reduced by 45% to 96% when compared to milking without teat-cleaning (Magnusson et al. 2006). Some teat-cleaning methods involve treatment with solutions, foams or wetted towels containing disinfectants. These methods to some extent inactivate vegetative bacteria, but no evidence is currently available that spores are inactivated. Other theoretical contamination pathways, such as aerial contamination of raw milk by spores from silage and direct contamination of milk by silage, are insignificant under normal production conditions (Vissers et al. 2006, Vissers 2007).

Vissers et al. (2006) developed a predictive model of the contamination of raw milk by spores of butyric acid bacteria from feed or other sources in the farm environment, for instance soil. The model was based on a mathematical translation of the contamination pathway described above. It was used to evaluate the most important variables and to identify effective and non-effective strategies to control levels of spores of butyric acid bacteria in farm tank milk. It was concluded that the variation of the concentration of spores in silage is, by far, the most important variable, and significantly more important than, for instance, teat-cleaning efficiency and barn hygiene (see also Vissers 2007).

Clostridium species

Clostridium species that occur in silage have been summarized by Pahlow et al. (2003). These authors divided the most common species into three groups, based on their protein and carbohydrate fermentation properties (Table 1). The first group consists of so-called proteolytic clostridia, of which *Clostridium sporogenes* is the predominant species in silage. Species of this group derive their energy from fermentation of both proteins and carbohydrates. The second group was named the *Clostridium butyricum* group. Species of this group typically ferment a wide range of carbohydrates but are unable to ferment proteins. Klijn et al. (1995) showed that many strains originating from silage, the farm environment and milk and originally identified as *C. butyricum* on the basis of phenotypic characteristics, genetically belonged to the species *Clostridium beijerinckii*. The third 'group' is formed by *Clostridium tyrobutyricum*, which ferments a limited number of carbohydrates but in addition has the ability to ferment lactic acid to acetic acid and butyric acid at low pH. This type of fermentation is known as butyric acid fermentation and the bacteria responsible for it are referred to as butyric acid bacteria. In addition to the above-mentioned species, Rossi and Dellaglio (2007) detected *Clostridium saccharolyticum* and *Clostridium baratii* in silages with high counts of clostridial spores. Using a cultivation-independent DNA-based method (PCR-denaturing gradient gel electrophoresis; DGGE), Julien et al. (2008) identified *Clostridium disporicum* as another predominant member of clostridial populations in silage.

C. tyrobutyricum is a species that is studied most in relation to silage quality for two reasons. Firstly, because *C. tyrobutyricum* is the main cause of butyric acid fermentation in silage due to its tolerance to low pH conditions in combination with its ability to use lactic acid as a substrate for growth (Pahlow et al. 2003, Vissers 2007). This can have large negative effects on the preservation quality, nutritive value and palatability of silage. The second reason is that spores of *C. tyrobutyricum* that are present in silage are transferred to milk, and their presence in cheesemilk can lead to a defect called late-blowing in semi-hard and hard cheese types, such as Gouda, Emmental and Gruyère. Late-blowing is caused by butyric acid fermentation that takes place during cheese ripening and results in off-flavours and excessive gas formation leading to texture defects. Late-blowing may cause significant loss of product. Interestingly, the factors that are important for growth of *C. tyrobutyricum* in silage and cheese are the same: low pH, low water activity, use of lactic acid as a substrate and a low concentration of nitrate. In both silage and cheese a high level of nitrate inhibits the germination of spores and outgrowth of *C. tyrobutyricum* (Klijn et al. 1995, Pahlow et al. 2003). Studies by Klijn et al. (1995) showed that late-blowing in Gouda cheese is exclusively associated with growth of *C. tyrobutyricum*. Cheese made from milk in which spores of other silage-associated *Clostridium* species, such as *C. beijerinckii* and *C. sporogenes*, were added showed no signs of late-blowing. Moreover, growth of *C. tyrobutyricum* was detected in all experimental and commercial cheeses showing obvious signs of late-blowing. Since *C. tyrobutyricum* is not harmful to man and animals, its occurrence in silage and cheese is only of economic importance.

Table 1. The predominant *Clostridium* species occurring in silage and their characteristics. Adapted from Pahlow et al. 2003. Based on data from Ali-Yrkkö and Antila 1975, Bühler 1985, Klijn et al. 1995, Rossi and Dellaglio 2007, Julien et al. 2008.

Characteristic	Proteolytic group	*C. butyricum* group	*C. tyrobutyricum*
Species	*C. sporogenes*	*C. butyricum*	*C. tyrobutyricum*
	C. bifermentans	*C. beijerinckii*	
	C. baratii	*C. acetobutyricum*	
		C. saccharolyticum	
		C. disporicum	
Minimum pH allowing growth	>5	>4.5	>4.2
Substrates fermented:			
Proteins	+	-	-
Carbohydrates	+	+	+
Monosaccharides	variable	many	few
Lactate	weak	-	+

The pathogen *Clostridium botulinum*, the causative agent of botulism, is rarely found in silage. Botulism is caused by highly potent neurotoxins produced by *C. botulinum* (botulinum toxins). Occurrence of *C. botulinum* and botulinum toxins in silage can be associated with the presence of carcasses of birds or small mammals, for instance due to killing of the animals during harvesting of the crop (Cobb et al. 2002). Poultry manure is a notorious source of spores of *C. botulinum*. Silage crops may become contaminated with *C. botulinum* spores when contaminated poultry manure is used as a fertilizer (Livesey et al. 2004). *C. botulinum* is more sensitive to low pH values than for instance *C. tyrobutyricum* and *C. beijerinckii* (Pahlow et al. 2003). For that reason, *C. botulinum* does not grow in silage under normal ensiling conditions. Occurrence of *C. botulinum* in silage and its relevance in cattle have been reviewed previously (Kehler and Scholz 1996, Lindström et al. 2010). Foodborne botulism occurs when foods are consumed in which botulinum toxins have been formed. Foods associated with foodborne botulism include canned vegetables and low-acid foods (in particular home-canned foods), sausages, meat products and seafood products (Sobel et al. 2004). Because of their occasional occurrence in silage, transfer of *C. botulinum* spores to raw milk cannot be excluded. However, historically, dairy products have not been associated with outbreaks of foodborne botulism (Shapiro et al. 1998, Sobel et al. 2004).

The concentration of spores of butyric acid bacteria is traditionally determined by most probable number (MPN) methods, using (1) a medium containing lactic acid and incubation conditions that are selective for anaerobic, gas-forming bacteria, and (2) pasteurization of sample dilutions before inoculation of the medium to inactivate vegetative bacteria (Bergère and Sivelä 1990). These methods are useful for enumeration of *C. tyrobutyricum* spores, but they are not specific for this species. Other species, for instance *C. beijerinckii*, are sometimes detected as well. Detection of butyric acid bacteria spores in raw milk is part of the milk quality systems of a number of dairy companies, for instance Dutch dairy companies. Apart from not exclusively detecting *C. tyrobutyricum*, MPN

methods for enumeration of butyric acid bacteria spores have several other disadvantages: the analysis time is long (4 to 7 days) and the results have a high uncertainty (which is inherent to most MPN procedures). Alternative methods for detection of *C. tyrobutyricum* are available, for instance methods based on qPCR (Herman et al. 1995, Lopez-Enriquez et al. 2007) or immunological techniques (Nedellec et al. 1992, Lavilla et al. 2010). However, these methods are currently not used for routine analyses in the dairy sector, presumably because they are relatively laborious and costly.

Concentrations of butyric acid bacteria spores in silage vary from 10 to 100 spores g^{-1} fresh matter, which represents the initial contamination level of fresh crops arising from soil contamination at harvest, to 10^6 to 10^7 spores g^{-1} in silages with extensive butyric acid fermentation (Stadhouders and Spoelstra 1990, Pahlow et al. 2003, Vissers et al. 2007b). Information about concentrations of butyric acid bacteria spores in farm-scale silages is rather scarce. Studies in France conducted in the 1970s showed that about 20% of grass silages contained more than 10^5 butyric acid bacteria spores g^{-1}, a level that can be described as 'poor quality' (ITEB/ITG 1980). In a survey conducted in the Netherlands in 1982, 44% of grass silages exceeded the level of 10^5 butyric acid bacteria spores g^{-1} and the average concentration was 4.9 $\log_{10}$ g^{-1} (Spoelstra 1984, 1990). More recent data from the Netherlands show that the quality of grass silage with regard to butyric acid bacteria spores has significantly improved since the 1980s: in 5% of grass silages produced between 2002 and 2004 the concentration of butyric acid bacteria spores exceeded 10^5 g^{-1} and the average concentration was 3.2 $\log_{10}$ g^{-1} (Table 2). These results were based on samples taken from unopened silages sampled, on average, eleven weeks after ensiling. The improvement of silage quality with regard to butyric acid bacteria spores in the Netherlands since the 1980s is in agreement with information from Dutch dairy companies, who experienced a decreasing trend in the level of butyric acid bacteria spores in raw milk delivered by Dutch farmers between 1980 and 2000. The survey conducted in the Netherlands between 2002 and 2004 also included samples from unopened maize silages. The average concentration of butyric acid bacteria spores in maize silage were approximately 0.5 $\log_{10}$ unit lower than in grass silage and maize silages, with a high level of butyric acid bacteria spores almost absent: none of 197 tested maize silages exceeded 10^5 spores g^{-1} and only 0.5% exceeded 10^4 spores g^{-1} (Table 2). The findings were in accordance with the knowledge that, due to the low buffering capacity of the maize, lactic acid fermentation in maize silage is generally fast and the final pH low (pH 3.8 to 4.0), conditions that do not favour outgrowth of butyric acid bacteria.

Table 2. Concentration of butyric acid bacteria spores in unopened and opened grass and maize silages and mixed silage at commercial dairy farms in the Netherlands. From unopened silages, only core samples were analyzed. From opened silages, samples from the core, surface layer and areas with visible moulds were analyzed. Mixed silage consisted of grass and maize silage and was sampled in the barn where it was offered to dairy cows. Data from Vissers et al. (2007a), Vissers et al. (2007b) and Vissers and Driehuis, unpublished results.

Sample type	Number of samples	Average concentration ($\log_{10}$ spores g^{-1})	Percentage of samples containing (spores g^{-1}):			
			$<10^3$	10^3-10^4	10^4-10^5	$>10^5$
Grass silage, unopened	460	3.2	48%	32%	15%	5%
Grass silage, opened						
Core	22	3.0	50%	41%	9%	0%
Surface layer	22	3.1	64%	23%	9%	5%
Area with visible moulds	14	3.9	21%	29%	29%	21%
Maize silage, unopened	197	2.7	79%	21%	0.5%	0%
Maize silage, opened						
Core	21	3.0	62%	19%	14%	5%
Surface layer	21	3.6	43%	24%	14%	19%
Area with visible moulds	15	5.5	7%	13%	13%	67%
Mixed silage in barn	122	4.2	17%	24%	41%	18%

Until recently, the generally accepted view was that high concentrations of butyric acid bacteria spores are associated with anaerobic instability of silage due to insufficient pH decline during the primary fermentation phase and that growth of clostridia in silage depends on the contents of dry matter, water soluble carbohydrates and nitrate and the buffering capacity of the crop before ensiling (McDonald et al. 1991, Kaiser et al. 2002, Pahlow et al. 2003). A different view on the issue of butyric acid bacteria spores in silages came from a study by Vissers et al. (2007a), who showed that, on Dutch dairy farms, increased concentrations of butyric acid bacteria spores were often related to aerobic instability problems rather than to anaerobic instability problems. In that study, samples

were taken at 21 commercial dairy farms from various locations in clamp silos of grass and maize silage and from mixed grass and maize silage that was offered to dairy cows in the barn. It was found that the samples of mixed silage in the barn had an average concentration of butyric acid bacteria spores that was more than 10-fold higher than samples taken from the core of the grass and maize silage clamps, which represented the major fraction of the silage that was offered to cows. In addition, it was found that a high percentage of samples of mixed silage (18%) contained butyric acid bacteria spores in a concentration exceeding 10^5 spores g^{-1} (Table 2). The study showed that the total quantity of butyric acid bacteria spores consumed by cows was determined by only a small fraction of silage that contained a high concentration of above 10^5 spores g^{-1} ('hot spots'). Further analysis of the silages that were used at the farms revealed that high spore concentrations were detected particularly in samples from areas showing signs of aerobic deterioration, i.e. areas with a high concentration of yeasts and moulds and increased temperature and pH. High concentrations of butyric acid bacteria spores were found most often in surface layers and in particular in areas with visible moulds (up to 10^7 spores g^{-1}). Unexpectedly, high concentrations of butyric acid bacteria spores were detected more often in maize silage than in grass silage (Table 2). The data showed that at the surveyed farms, which were representative for dairy farming in the Netherlands, maize silage contributed more to the total intake of butyric acid bacteria spores by dairy cows than grass silage. The results by Vissers et al. (2007a) confirmed earlier observations by Jonsson (1989, 1991), who showed that *C. tyrobutyricum* has the ability to grow and produce spores in silage that is exposed to air. Also recent studies from Italy indicated that high levels of clostridia spores in maize silage are associated with air penetration and aerobic deterioration processes (Borreani and Tabacco 2008, 2010).

The growth of the strictly anaerobic bacterium *C. tyrobutyricum* in aerobically deteriorated areas of silage may seem contradictory. However, microbial ecosystems with aerobic and anaerobic zones are found in many environments, for example in sediments and intestines (Brune et al. 1995, Fourcans et al. 2004). The occurrence of anaerobic niches in aerobically deteriorating silage was postulated for the first time by Jonsson (1989). The occurrence of these niches may be explained as follows. Aerobic deterioration in silage areas that are exposed to air is usually initiated by growth of acid-tolerant, lactate-assimilating yeasts that oxidize residual sugars and organic acids, leading to an increase in pH. Since the concentration of oxidizing yeasts is relatively low during the early phases of aerobic deterioration, the consumption rate of oxygen is also low and oxygen penetrates relatively deep into the silage. However, as the concentration of the yeasts increases, the consumption rate of oxygen also increases. As a result, oxygen penetrates less deeply into the silage, and deeper parts of the silage return to anaerobic conditions (Muck and Pitt 1994). Consequently, anaerobic niches with an increased pH may develop close to air-exposed areas and growth of *C. tyrobutyricum* and possibly other clostridia are no longer inhibited due to the increased pH in these niches.

Bacillus cereus and other aerobic spore-forming bacteria

Spores of aerobic spore-forming bacteria are ubiquitous and can be isolated from a wide variety of sources in the dairy farm environment, including soil, silage, concentrate feeds, bedding and faeces. Contamination of raw milk by spores from these sources occurs during milking via contaminated udders and teats, as described earlier in this paper. After the initial contamination, the concentration of spores may increase further during storage of the milk at the farm, for instance when the storage temperature is not low enough and spores of psychrotrophic spore-forming bacteria germinate. Another possible route of contamination is via insufficiently cleaned milking equipment. Certain spore-formers are known to be capable of forming biofilms which can attach to stainless steel and release high numbers of spores into surpassing milk (Eneroth et al. 2001, Simoes et al. 2010).

Aerobic spore-formers with particular relevance for dairy products are *Bacillus cereus* and the highly heat-resistant spore-formers *Bacillus sporothermodurans* and *Geobacillus stearothermophilus*. *B. cereus* is a major spoilage organism of pasteurized milk and milk products stored at refrigeration temperature (Griffiths 1992, Te Giffel 1997, Heyndrickx and Scheldeman 2002). *B. cereus* spores also occur in milk powder and in infant formulae that contain milk powder. Spores of psychrotrophic strains of *B. cereus* are capable of germination and the bacteria can grow in pasteurized milk and milk products at temperatures as low as 5°C. The content of spores of psychrotrophic *B. cereus* often limits the shelf life of these products as high levels may cause off-flavours and curdling. *B. cereus* is also a concern for food safety as it can produce different types of toxins and is a potential food poisoning agent (Stenfors Arnesen et al. 2008). Therefore, the organism is generally regarded as a pathogen. Dairy products are only sporadically involved in outbreaks of foodborne illness caused by *B. cereus*. *B. sporothermodurans* and *G. stearothermophilus* are thermophilic bacteria producing highly heat-resistant spores and can cause non-sterility problems in ultrahigh-temperature (UHT) processed or sterilized milk products (Huemer et al. 1998, Scheldeman et al. 2006). The section below focuses on the role of silage as a source of spores of aerobic spore-formers in raw milk.

Aerobic spore-formers isolated from silage belong to the taxonomic families *Bacillaceae* and *Paenibacillaceae* (Table 3). Species frequently isolated include *B. cereus*, *B. licheniformis*, *B. coagulans*, *B. pumilus*, *B. sphaericus* and *Paenibacillus polymyxa*. Populations of heat-resistant spore-formers that are isolated from silage show high diversity and also include *B. sporothermodurans*, the species associated with spoilage of UHT-products. The primary source of most aerobic spore-formers is soil (Claus and Berkeley 1986). Concentrations of spores of aerobic spore-formers and spores of *B. cereus* in soil vary from 10^5 to 10^7 spores g^{-1} and 10^1 to 10^6 spores g^{-1}, respectively, depending on soil type, sampling site and season (Rammer et al. 1994, Slaghuis et al. 1997, Christiansson et al. 1999, Vissers et al. 2007c, Vissers and Driehuis, unpublished data). The actual levels of spores of aerobic spore-formers and spores of *B. cereus* on crops prior to ensiling depend on the amount of soil that contaminates the crop during growth in the field and during harvesting. They also depend on whether the soil or crop has been fertilized with cattle manure, since spore concentrations can be high in cattle faeces. Slaghuis et al. (1997) detected a concentration of spores of aerobic spore-formers in grass and maize prior to ensiling of 10^2 to 10^4 spores g^{-1}. Several studies, summarized by Pahlow et al. (2003), have reported on concentrations of spores of aerobic spore-formers occurring in farm-scale silages. The reported spore concentrations vary considerably between the silos. For wilted grass silages these concentrations ranged from 10^3 to 10^8 spores g^{-1}, for whole crop maize silages from 10^2 to 10^9 spores g^{-1} and for sugar beet pulp and brewers' grain silages from 10^3 to 10^7-10^8 spores g^{-1}. Concentrations in core samples of silages were generally on the lower side of these concentration ranges: 10^4 to 10^5 spores g^{-1} in grass silages and 10^3 to 10^4 spores g^{-1} in maize silages. These data are in line with the view that in well-fermented silage germination of spores and outgrowth of vegetative bacteria does not occur. Growth of aerobic spore-formers probably occurs during the later phases of aerobic deterioration, *i.e.* after aerobic deterioration has been initiated by yeasts or acetic acid bacteria (Pahlow et al. 2003). High levels of spores of aerobic spore-formers have been detected in the surface layers of grass and maize silage (Slaghuis et al. 1997, Driehuis et al. 2009). Table 4 summarizes results from studies in the Netherlands on concentrations of spores of aerobic spore-formers in unopened and opened farm-scale grass and maize silages and on the distribution of spores in opened silages. The highest levels were detected in surface layers and areas with visible moulds of opened silages and in mixed grass and maize silage offered to cows in the barn. These data confirm that high concentrations of spores of aerobic spore-formers relate to aerobic deterioration problems.

Table 3. Species of aerobic spore-forming bacteria isolated from silage. Data from Lindgren et al. 1985, Jonsson 1989, McDonald et al. 1991, De Silva et al. 1998, Inglis et al. 1999, Pettersson et al. 2000, Te Giffel et al. 2002, Driehuis et al. 2009.

	Species
Heat-resistance not specified	*Bacillus cereus*, *Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus pumilus*, *Bacillus sphaericus*, *Bacillus firmus*, *Bacillus lentus*, *Bacillus circulans*, *Paenibacillus polymyxa*, *Paenibacillus validus*, *Paenibacillus pabuli*, *Brevibacillus chosinensis*
Highly heat-resistant species	*Bacillus cereus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus sporothermodurans*, *Bacillus oleronius*, *Bacillus siralis*, *Brevibacillus borstelenis*, *Aneurinibacillus* spp.

Table 4. Average pH and microbiological composition of samples from unopened and opened grass and maize silages and from mixed grass and maize silage offered to cows at commercial dairy farms in the Netherlands (Driehuis et al. 2009). The data were collected between 2002 and 2005.

Sample type	Number of samples	Spores of aerobic spore-formers (log_{10} spores g^{-1})	Yeasts & moulds (log_{10} cfu g^{-1})	pH
Grass silage				
Unopened, core	460	4.8	3.8	4.8
Opened, core	22	5.1	3.3	4.8
Opened, surface	22	5.7	4.9	5.3
Opened, area with visible moulds	14	8.0	6.8	7.1
Maize silage				
Unopened, core	197	3.3	6.0	3.8
Opened, core	21	4.5	5.8	3.9
Opened, surface	21	5.1	7.0	4.2
Opened, area with visible moulds	15	7.7	7.8	6.6
Mixed silage in barn[a]	122	6.2	6.4	4.8

[a] Mixed grass and maize silage offered to dairy cows in the barn

As described previously, spores of *B. cereus* occur in silages. However, this species does not increase in numbers to the same extent as other aerobic spore-formers. Vissers et al. (2007c) monitored *B. cereus* spore concentrations in different feeds, faeces, bedding, soil and raw milk at 24 commercial Dutch dairy farms and detected average concentrations of *B. cereus* spores of 2.2 to 2.8 $\log_{10}$ spores g^{-1} in mixed silage offered to cows with maximum concentrations of 4.0 $\log_{10}$ spores g^{-1}. Although feed in general, and silages in particular, were found to be important sources of *B. cereus* spores in raw milk, it was concluded that the concentrations detected in silage were not critical with respect to the quality and safety of dairy products. The authors indicated that soil and insufficiently cleaned milking equipment are more critical potential sources of *B. cereus* spores at dairy farms. In studies conducted in Sweden, soil was identified as the major source of contamination of raw milk by *B. cereus* during grazing of cows, whereas used sawdust bedding material, in particular in free-stalls with deep sawdust beds, was a major source when cows were kept indoors (Christiansson et al. 1999, Magnusson et al. 2007).

Listeria monocytogenes

The facultatively anaerobic Gram-positive bacterium *Listeria monocytogenes* is an important food-borne pathogen because it is the causative agent of listeriosis. Due to the severity of this disease, the high mortality rate and the increasing incidence, *L. monocytogenes* is of great concern to public health (European Food Safety Authority 2011). The bacterium is widely distributed in the environment and has been isolated from a variety of sources, including soil, surface water and vegetative materials. It occurs at low numbers in many raw and ready-to-eat foods. Consequently, humans are commonly exposed to low numbers of *L. monocytogenes* from various types of food. Generally, this is not considered a serious health hazard (Food and Drug Administration 2001). However, ingestion of food contaminated with high numbers of *L. monocytogenes* may result in disease, in particular in populations with an increased risk of listeriosis, such as immunocompromised patients, elderly and neonates. High numbers of *L. monocytogenes* in foods usually arise from growth during storage of contaminated food products that support the growth of the bacterium. Foods particularly linked to *L. monocytogenes* contamination include raw and smoked fish, raw and cooked meat, and soft and semi-soft cheeses produced from unpasteurised milk. For these and other ready-to-eat foods that are able to support the growth of *L. monocytogenes* the internationally applied food safety criterion is absence in 25 g throughout their shelf life (also referred to as zero-tolerance). For foods that are unable to support the growth of *L. monocytogenes* and foods in which limited growth can occur a commonly applied criterion is 100 cfu g^{-1} during their shelf life (see for instance EU regulation on microbiological criteria for foodstuffs; European Commission 2005).

An important feature of *L. monocytogenes* is its psychrotolerance. The bacterium has the ability to grow at temperatures as low as 0 °C, and therefore can grow during refrigerated storage of foods (Wilkins et al. 1972). The bacterium also has considerable osmotolerance and acid tolerance, although it is unable to grow at pH levels lower than 4.4. Due to its high tolerance to stress conditions, *L. monocytogenes* is capable of survival for extended periods in environments in which it is unable to grow. As a vegetative bacterium, *L. monocytogenes* is fairly sensitive to heat inactivation and the organism is effectively killed by pasteurization processes of milk that are used in the dairy industry. Therefore, heat treatment is an effective processing tool in the control of *L. monocytogenes* in foods. Contamination of processed food products by *L. monocytogenes* often results from recontamination during the manufacturing process or packaging, and environments inside food processing plants have been recognized as important potential sources of *L. monocytogenes* (Wiedmann 2003).

Outbreaks and sporadic cases of listeriosis in cattle, sheep and goats have been associated with feeding of silage contaminated with *L. monocytogenes* (Fenlon, 1988, Ho et al. 2007). Different studies have shown a high diversity of *L. monocytogenes* strains in silages and in faeces shed by cows that were fed silage (Nightingale et al. 2004, Borucki et al. 2005). Not only animals with clinical signs of listeriosis shed *L. monocytogenes* in their faeces. Asymptomatic animals from farms with an outbreak of listeriosis and healthy animals from farms without a record of listeriosis cases can shed the bacterium also (Unnerstad et al. 2000, Nightingale et al. 2004, Vilar et al. 2007). Contamination of raw milk by *L. monocytogenes* has been linked to the occurrence of high levels of *L. monocytogenes* in silage (Sanaa et al. 1993, Tasci et al. 2010). Transmission of *L. monocytogenes* to raw milk is most likely taking place via faeces and bedding that is contaminated by faeces, as described previously for bacterial spores. Another transmission route is shedding of *L. monocytogenes* in milk by cows with mastitis caused by this bacterium (Bourry et al. 1995). However, the incidence of bovine mastitis caused by *L. monocytogenes* is low (Fedio et al. 1990).

The degree of anaerobiosis and the pH are important factors determining survival and growth of *Listeria* spp. in silage. *L. monocytogenes* added to grass at ensiling rapidly disappeared under strictly anaerobic conditions and

at a pH lower than 4.4. However, at an oxygen tension of 0.5% (v v^{-1}) survival was prolonged, and growth was observed even at a pH as low as 4.2. Higher oxygen tensions strongly encouraged *L. monocytogenes* growth (Donald et al. 1995). High numbers of *L. monocytogenes* and other *Listeria* species have been detected in different types of silage. For instance, *L. monocytogenes* levels in excess of 10^6 cfu g^{-1} were detected in surface layers of big bale grass silages that were visibly infested by moulds (Fenlon 1986). Different studies have shown that the incidence of *Listeria* species in silage increases with increasing pH (Ryser et al. 1997, Vilar et al. 2007, Tasci et al. 2010). For instance, Vilar et al. (2007) detected *Listeria* spp. in 30% of silage samples with a pH ≥4.5 and in 6% of samples with a pH <4.5. These data are in line with the view that the occurrence of *Listeria* species in silage is associated with aerobic deterioration problems. The relatively high pH values that generally exist in aerobically deteriorated areas, in combination with the presence of oxygen lead to conditions that favour growth of *Listeria*. Silages with a greater likelihood of aerobic surface spoilage are more susceptible to contamination by *Listeria*, for example silage with low packing density, silage that is inadequately sealed and big bale silage (Fenlon et al. 1989).

In conclusion, *L. monocytogenes* has frequently been detected in silages and has been associated with occurrence of aerobic spoilage. Its presence in silage has been linked to contamination of raw milk. However, since *L. monocytogenes* is effectively inactivated by pasteurization used in milk processing, the food processing plant environment appears to be the major source of finished product contamination.

Enterobacteriaceae and *Escherichia coli*

Several species of the facultatively anaerobic *Enterobacteriaceae* belong to the epiphytic microflora of most forage crops. *Erwinia herbicola* and *Rahnella aquitilis* often dominate the fresh crop, but after ensiling these species are rapidly superseded by other species, such as *Hafnia alvei*, *Escherichia coli* and *Serratia fonticola* (Heron et al. 1993). The most important species in this group from the viewpoint of human health risks is *E. coli*. Most *E. coli* strains are harmless and are part of the normal intestinal microbiota of humans and many animals. However, some types of *E. coli* cause severe gastrointestinal diseases. Among the pathogenic *E. coli*, the group of Shiga toxin-producing *E. coli* (STEC), also called verocytotoxin-producing *E. coli* (VTEC) or enterohaemorrhagic *E. coli* (EHEC), is of serious public health concern.

The gastrointestinal tract of healthy ruminants, including cattle, is recognized as the main natural reservoir of STEC, in particular for *E. coli* O157:H7. Major sources of *E. coli* O157:H7 and other STEC strains for human infection are (raw) meat products, faecally contaminated vegetables and drinking water, and direct contact with animals. In addition, raw milk and unpasteurized dairy products have been implicated in outbreaks caused by infection with *E. coli* O157:H7 (Hussein and Sakuma 2005). The presumed route of transmission to raw milk is faecal contamination during milking, as described previously for bacterial spores and *L. monocytogenes*. Fortunately, *E. coli* O157:H7, like other *E. coli*, is sensitive to heat and is effectively killed by pasteurization of milk used in the dairy industry. Therefore, as described previously for *L. monocytogenes,* heat treatment is an effective processing tool in the control of *E. coli* O157:H7 and other STEC strains in dairy products.

During the early stages of silage fermentation, *Enterobacteriaceae* compete with the lactic acid bacteria and other bacterial groups for nutrients. Most *Enterobacteriaceae* do not grow and lose viability at pH values lower than 4.5 to 5.0. A fast pH decline therefore decreases growth and survival of *Enterobacteriaceae* in silage (Heron et al. 1993). However, the presence of oxygen prolongs their survival in silage and some enterobacteria that survive the storage phase may start growing again and reach numbers in excess of 10^8 cfu g^{-1} when silage pH increases during aerobic deterioration (Lindgren et al. 1985, Donald et al. 1995). No studies are known to the author that have shown the presence of *E. coli* O157:H7 and other STEC strains in silage. In a number of studies the growth and survival of *E. coli* O157:H7 in grass, maize and barley silage, inoculated with this bacterium prior to ensiling, was investigated. These studies showed that *E. coli* O157:H7 does not survive in well-fermented silage with a fast pH decline and low pH (Byrne et al. 2002, Bach et al. 2002, Pedroso et al. 2010). The same result was achieved for STEC serotype O26 in maize silage (Dunière et al. 2011). However, other studies showed that *E. coli* O157:H7 potentially can survive and grow in poorly fermented silage and in aerobically deteriorated silage (Fenlon and Wilson 2000, Pedroso et al. 2010).

In conclusion, *E. coli* O157:H7 and other STEC strains do not survive normal ensiling conditions and no data showing occurrence of these bacteria in silages are currently available.

Mycotoxins

This section summarizes scientific knowledge about the major mycotoxins occurring in silages, the conditions under which they are formed and prevention of their formation. Mycotoxins in silage are of dual concern. Firstly, they can have adverse effects on animal health and cause production losses. Secondly, they may jeopardize the safety of food products of animal origin. Of the major mycotoxins in silage crops, the second concern holds true for aflatoxin only, as is described further on in this paper. The metabolism of mycotoxins in ruminants and their carry-over into milk are briefly described. Toxic effects of mycotoxins in animals and man, analytical methods for detection of mycotoxins and legislative aspects are not described in this paper. Information about these topics can be found elsewhere (Council for Agricultural Science and Technology [CAST] 2003, Krska et al. 2008, Driehuis et al. 2010).

Mycotoxins are a large, diverse group of toxic metabolites of fungi. Currently, more than 300 mycotoxins have been identified (CAST 2003). Mycotoxins can be found in a wide variety of crops all around the world, including crops that are commonly fed as silage, such as maize, wheat and grasses (CAST 2003, Driehuis et al. 2010). Moulds and mycotoxins of relevance for silage that is produced from these crops are listed in Table 5. A distinction is made between mycotoxins that are formed before ensiling and those that are formed after ensiling. It is important to make this distinction because different types of moulds, different types of mycotoxins and different types of agricultural factors influencing mycotoxin levels are involved. Mycotoxins that are formed before ensiling are associated with moulds that infect a crop during its growth in the field or with endophytic moulds that live as symbionts in for instance grasses or cereals (field-derived mycotoxins). Field-derived mycotoxins include trichothecenes, zearalenone, fumonisins, aflatoxins and ergot alkaloid mycotoxins. Mycotoxins that are formed after ensiling are associated with moulds that develop in silage during storage or feeding-out (ensilage-derived mycotoxins), usually as a result of poor silage management practices. These mycotoxins include mycotoxins formed by *Penicillium roqueforti* and *Penicillium paneum* and a diverse group of mycotoxins formed by *Aspergillus fumigatus*.

Table 5. Major mycotoxigenic moulds and mycotoxins in silage crops and silages.

Mycotoxin group	Major toxin(s)	Mould species	Crop(s)	Field- or ensilage-derived
Aflatoxins	Aflatoxin B_1 (M_1), B_2, G_1, G_2	*Aspergillus flavus, A. parasiticus*	Maize	Field
Trichothecenes	Type A: T_2, diacetoxyscirpenol	*Fusarium langsethiae, F. poae, F. sporotrichioides*	Maize, Sg cereals[1]	Field
	Type B: DON, nivalenol	*F. graminearum, F. culmorum*	Maize, Sg cereals, grass	Field
Fumonisins	Fumonisin B_1, B_2	*F. verticillioides, F. proliferatum*	Maize	Field
Resorcylic acid lactones	Zearalenone	*F. graminearum, F. culmorum*	Maize, Sg cereals, grass	Field
Ochratoxins	Ochratoxin A	*A. ochraceus, Penicillium verrucosum*	Sg cereals	Field
Ergot alkaloids	Clavines, lysergic acid amide, ergotamine	*Claviceps purpurea*	Sg cereals	Field
	Lolitrem B, ergovaline	*Neotyphodium lolii, N. coenophialum*	Grass	Field
P. roqueforti toxins	Roquefortine C, mycophenolic acid	*P. roqueforti, P. paneum*	All types of silages	Ensilage
A. fumigatus toxins	Gliotoxin, fumigaclavines	*A. fumigatus*	All types of silages	Ensilage
M. ruber toxins	Monacolin K, citrinin	*Monascus ruber*	All types of silages	Ensilage

[1] Sg cereals: Small grain cereals (wheat, triticale, rye, barley).

Field-derived mycotoxins

The major toxinogenic moulds capable of producing field-derived mycotoxins are *Fusarium* species, *Aspergillus flavus* and *Aspergillus parasiticus* and endophytic *Claviceps* and *Neotyphodium* species. Detailed information about these moulds and mycotoxins can be found elsewhere (CAST 2003, Barug et al. 2006, Driehuis et al. 2010). The most frequently occurring mycotoxins produced by *Fusarium* species are trichothecenes, zearalenone and fumonisins. These moulds occur world-wide, but seem to be particularly prevalent in temperate climates. The development of *Fusarium* mycotoxins is strongly influenced by weather conditions. Infection of plants by *Fusarium* can take place via kernels, leaves, the stalk or infected seeds. Soil and decaying plant residues in the field are the

main sources of *Fusarium* spores and conidia. A high level of mechanical or insect damage of the plant increases the risk of infection and is often associated with higher mycotoxin levels.

Examples of plant diseases associated with *Fusarium* infection include ear rot and stalk rot in maize and ear blight in wheat. The predominant species causing these diseases are *Fusarium graminearum* and *Fusarium culmorum*. These species are capable of producing zearalenone and different types of trichothecenes, including deoxynivalenol (DON; synonym for vomitoxin), nivalenol, diacetoxyscirpenol and T-2 and HT-2 toxin. DON is the most commonly occurring trichothecene. DON and zearalenone often co-occur in contaminated crops. An important and often unrecognized feature of DON and zearalenone contamination of maize and wheat is that these mycotoxins occur not only in the grains and kernels but also in the green parts of the plant, *i.e.* the leaves and stalk. This is of significance because these crops are often fed as whole crop silage. The limited information that is available on this topic indicates that DON and zearalenone levels can be even higher in leaves and stalk of maize than in the cob (Oldenburg et al. 2005). Fumonisins are formed by *Fusarium verticillioides* (syn. *Fusarium moniliforme*) and *Fusarium proliferatum*, species associated with pink or white ear rot disease in maize. Fumonisins are found exclusively in maize. It is generally assumed that DON, zearalenone and other *Fusarium* mycotoxins are not produced in silage (Driehuis et al. 2010). *Fusarium* species do not survive the acidic and anaerobic conditions of silage and usually have a lower prevalence in silage than for instance *Aspergillus*, *Penicillium* and *Monascus* species. However, a few studies have reported development of *Fusarium* mycotoxins in silage. An example is a study conducted in Italy in which zearalenone was detected in high concentrations in extensively aerobically deteriorated peripheral areas of maize silage. Concentrations in these areas were up to 40 times higher than concentrations in non-deteriorated central areas of the silage, which were similar to the concentration of the forage at ensiling (Cavallarin et al. 2004).

Aflatoxins are produced by *A. flavus* and, to a lesser extent, *A. parasiticus*. Aflatoxins are highly toxic and carcinogenic to man and animals. Aflatoxin B_1 is the most prevalent and most toxic form. Aflatoxin B_1 is transformed into aflatoxin M_1 in the liver of cattle. In this form it is (partially) excreted into milk. With respect to the risks of mycotoxins in feed aflatoxin M_1 is the only mycotoxin of concern for the safety of dairy products to consumers. This relates to its significant feed-to-milk carry-over rate and its high toxicity. Although *Aspergillus* is generally classified as a mould associated with mycotoxin production during storage of commodities, it can infect crops in the field under favourable conditions, especially in subtropical and warm temperate climates. *A. flavus* and *A. parasiticus* are associated with aflatoxin production in a number of crops, including maize, sunflower, peanut and several tree nuts. Maize plants can become infected by *Aspergillus* conidia from the environment, usually soil or insects. A high level of insect damage increases the risk of infection. If conditions are favourable, the mould colonizes the cobs and penetrates into the kernels. Aflatoxin development in the kernels occurs within narrow ranges of moisture content and temperature. Drought stress generally increases aflatoxin development in maize.

Ergot alkaloid mycotoxins are produced by *Claviceps purpurea* in rye and barley and some grasses and by endophytic *Neotyphodium* moulds in perennial grasses. Detailed information about ergot alkaloid mycotoxins can be found elsewhere (Wyss et al. 1997, CAST 2003, EFSA 2012). *C. purpurea* infects the plant when flowering. It produces a resting structure, called sclerotia or ergots, that is comparable in size to grain kernels and allows the mould to survive adverse conditions. These ergots contain high concentrations of alkaloids (e.g. clavines and lysergic acid amide). Several grasses, such as perennial ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinacea*), can harbour endophytic *Neotyphodium* species capable of producing similar alkaloid mycotoxins (e.g. lolitrem B and ergovaline). The plant benefits from this symbiosis through increased drought tolerance and resistance to insects. Endophytic *Neotyphodium* are highly prevalent in 'wild' grass populations in natural or extensively managed pastures in the North America, Australia, New Zealand and Europe. The prevalence of mycotoxin-producing endophytes in intensively managed pastures is generally low. Grass cultivars selected for grazing or silage production often do not contain these types of endophytes.

Ensilage-derived mycotoxins

Since the majority of mould species are obligate aerobic micro-organisms, they do not develop in well-preserved, anaerobic silage. However, in practice, silages are not completely anaerobic. Firstly, because silage covering materials are generally not fully airtight. Secondly, because of unintended damage to the silage covering during storage (for instance caused by rodents or birds). Moreover, exposure to air becomes inevitable after the silo is opened for feeding. Growth of moulds and development of mycotoxins in silage are associated with the duration and extent of air infiltration. The extent of infiltration of air into the silage mass is mainly dependent on the porosity and density of the silage and the rate of silage removal after opening. The occurrence of moulds in silage is usually highest in surface layers.

Moulds that are commonly detected in silage are *P. roqueforti* and *P. paneum*, *Monascus ruber*, *A. fumigatus*, *Byssochlamys nivea*, *Mucoraceae* (in particular *Rhizopus nigricans*) and *Chrysonilia sitophila* (Scudamore and Livesey 1998, Pahlow et al. 2003). No mycotoxins from *Mucoraceae* and *C. sitophila* have been documented. The predominant mould species in silages is *P. roqueforti*, which is tolerant to acidic conditions and able to grow at oxygen levels as low as 0.1% (v v^{-1}) (Lacey 1989, Pahlow et al. 2003). At silage surfaces it usually forms white to grey coloured spots or layers. Occasionally, *P. roqueforti* forms typical green to blue coloured balls or lumps of mouldy silage approximately 50 to 100 cm below the top surface. This occurs particularly in maize silage. *P. paneum* is closely related to *P. roqueforti* and the occurrence of these species in silage cannot be differentiated visually (Boysen et al. 1996). *P. roqueforti* and *P. paneum* are capable of producing a wide range of mycotoxins *in vitro* under laboratory conditions, including for instance different roquefortines, mycophenolic acid, PR-toxin, festuclavine and agroclavine (Nielsen et al. 2006, O'Brien et al. 2006). *P. paneum* additionally produces patulin. However, a number of these mycotoxins are probably not formed in silage or may not be stable under conditions prevailing in silage (as discussed later in this paper). *A. fumigatus* is a mould species that is particularly detected in heavily moulded parts of silage and capable of producing a large number of different toxic metabolites, including gliotoxin, verruculogen, fumitremorgens, fumigaclavines and trypacidin (Richter et al. 2009). Apart from production of mycotoxins, the occurrence of *A. fumigatus* in silage is considered a health risk because inhalation of spores of this mould can cause lung disease (aspergillosis) in animals and man. *M. ruber* forms red-purple spots on silage surfaces and is a producer of monacolin K and citrinin (Schneweis et al. 2001). *B. nivea* is a producer of patulin (Escoula 1975).

Stability of mycotoxins in silage

Information about the stability of mycotoxins in silage is not fully conclusive and for some mycotoxins is contradictory. Possibly, this relates to the heterogeneity of silage and the fact that the conditions change over time. There are data indicating that certain field-derived and ensilage-derived mycotoxins are degraded in silage.

Zearalenone is generally regarded as stable in silage. No effect of ensiling on the zearalenone concentration was detected in studies in which the level of this mycotoxin was monitored for nine months of ensilage (Lepom et al. 1988, Garon et al. 2006). This finding is consistent with data showing that the average and range of zearalenone concentrations in maize silage and unfermented maize products used as feed ingredient are similar (Dänicke et al. 2000). With respect to the stability of DON in silage there is contradictory information. In a study investigating DON stability in wheat and maize silage it was concluded that ensiling induced a strong reduction of DON (Richter et al. 2009). However, no effect of ensiling on DON concentration was detected in other studies (Lepom et al. 1990, Garon et al. 2006). Furthermore, surveys in Europe and United States of America have shown a high incidence of DON in maize silages (see next section) and the average and range of DON concentrations are similar in maize silage and unfermented maize products used as feed ingredient (Dänicke et al. 2000). These data indicate that DON is stable in silage under most conditions or may be degraded to a limited extent. Aflatoxin B_1 produced in maize in the field has been found to be degraded slowly in maize silage (Kalac and Woolford 1982). This observation was confirmed in a recent French study, in which a 3-fold decline of aflatoxin B_1 was detected during nine months storage of maize silage (Garon et al. 2006). Likewise, partial degradation of ochratoxin A, a mycotoxin that is associated with small grain cereals, has been observed in ensiled barley (Rotter et al. 1990). No information is available about the stability of fumonisins in silage, but probably these mycotoxins are stable.

Contradictory information is available about the stability of ergot alkaloids produced by *Claviceps* and *Neotyphodium* species in silage. Health problems of cattle have been associated with high concentrations of ergovaline in silage from endophyte infected perennial ryegrass (Lean 2001) and with high concentrations of ergocryptine in silage from maize that was contaminated with a weed containing *Claviceps* ergots in the field (Naude et al. 2005). This indicates that these substances were at least partially stable in silage. On the other hand, the concentration of *C. purpurea* ergot alkaloids (ergometrine, ergotamine and ergocryptine) in extensively managed grasslands were strongly decreased when the grass was ensiled (Wyss et al. 1997). The *Penicillium* mycotoxins roquefortine C and mycophenolic acid appear to be stable in silage, whereas PR-toxin and patulin are presumably unstable. In contrast to roquefortine C and mycophenolic acid, PR-toxin and patulin are rarely detected in silage. Experiments with blue-veined cheeses manufactured with *P. roqueforti* strains showed that PR-toxin was degraded and detoxified as a result of a chemical reaction with ammonia and free amino acids (Scott and Kanhere 1979). Many types of silage contain relatively high concentrations of ammonia and free amino acids, so reaction with these compounds may be the reason that PR-toxin is often undetectable. For patulin a similar mechanism may apply. Patulin is known to react with SH-groups of cysteine and other sulphur containing amino acids in protein-rich environments and is known to be inactivated in fermented foods, such as wine, beer and cheese (Ciegler et al. 1976, Scott 1984).

Occurrence of mycotoxins in silage

Information about the incidence and concentrations of mycotoxins in silages is relatively scarce, in particular for silages other than maize silage. Table 6 gives an overview of results from surveys in Europe and the United States of America for DON and zearalenone in silage, conducted between 1989 and 2007. The data show that the incidence of DON in maize silage was high: in six out of seven surveys the incidence was 72 to 100%, in one it was 42%. The average DON concentration of positive samples in these surveys varied between 0.60 and 1.85 mg kg^{-1}. The incidence of zearalenone in maize silage was also high, but generally lower than that of DON: in five out of six surveys the incidence was 32 to 59%, in one it was 96%. The average zearalenone concentration of positive samples varied between 0.05 and 0.45 mg kg^{-1}. Information about the occurrence of DON and zearalenone in silages other than maize silage are scarce. In a survey in the Netherlands between 2002 and 2004, DON was not detected in 120 grass silage samples and in 3 of 30 (10%) wheat silages, whereas zearalenone was detected in 7 of the grass silages (6%) and none of the wheat silages (Driehuis et al. 2008a). Fumonisin contamination of maize is widespread, as indicated by the high incidence of fumonisins in maize and maize by-products intended for use in animal feed (Binder et al. 2007). Incidence of fumonisins in maize silage is also likely to be high, since evidence indicating degradation of fumonisins in silage is lacking. This is confirmed by the results of a survey in Midwestern USA in 2001 and 2002, in which fumonisin B_1, B_2, and B_3 were detected in, respectively, 97%, 72% and 57% of maize silages and average concentrations in positive silages were 0.615, 0.093, and 0.051 mg kg^{-1}, respectively (Kim et al. 2004). In contrast, in the survey in the Netherlands described earlier, fumonisin B_1 and B_2 were detected in only 1.4% of the maize silages (Driehuis et al. 2008a). This low incidence probably reflects that the environmental conditions of forage maize growth in the Netherlands are not favourable for infection by fumonisin producing moulds (*F. verticillioides*). Aflatoxin B_1 has been detected in maize silages in some surveys, but in most surveys this mycotoxin was undetectable in silages (Scudamore and Livesey 1998, Whitlow and Hagler 2005, Storm et al. 2008, Driehuis et al. 2008a). The occurrence of aflatoxins in silage is associated with geographical regions with a tropical or sub-tropical climate and is generally field-derived. However, there are reports indicating development of aflatoxins in poorly preserved silage with extensive mould infestation (Gonzalez Pereyra et al. 2008, Gonzalez Pereyra et al. 2011).

Table 6. Incidence and average and maximum concentrations of the *Fusarium* mycotoxins DON and zearalenone (ZEA) in silage in different surveys.

Mycotoxin	Silage crop	Location	Year(s)	Percentage positive (total number)[1]	Concentration (mg kg^{-1})[2]		Reference
					Average (of positive samples)	Maximum	
DON	Maize	North Carolina, USA	1989-1993	76% (106)	1.85	-	Whitlow and Hagler 2005
DON	Maize	Austria	1995-1999	91% (418)	0.75	2.8	Hochsteiner and Schuh 2001
DON	Maize	Germany	1998	79% (24)	1.61	9.86	Dänicke et al. 2000
DON	Maize	Pennsylvania, USA	2001-2002	42% (62)	0.6	3.7	Mansfield et al. 2005
DON	Maize	Netherlands	2002-2004	72% (140)	0.85	3.14	Driehuis et al. 2008a
DON	Maize	Netherlands	2005	100% (16)	0.93	2.39	Driehuis et al. 2008b
DON	Maize	Denmark	2007	100% (20)	1.06	5.09	Storm et al. 2010
DON	Wheat	Netherlands	2002-2004	10% (30)	0.62	1.17	Driehuis et al. 2008a
ZEA	Maize	North Carolina, USA	1989-1993	32% (93)	0.45	-	Whitlow and Hagler 2005
ZEA	Maize	Germany	1993-1995	38% (44)	0.05	0.17	Dänicke et al. 2000
ZEA	Maize	Austria	1995-1999	59% (149)	0.07	0.6	Hochsteiner and Schuh 2001
ZEA	Maize	Germany	1998	96% (24)	0.13	1.07	Dänicke et al. 2000
ZEA	Maize	Netherlands	2002-2004	49% (140)	0.17	0.94	Driehuis et al. 2008a
ZEA	Maize	Netherlands	2005	50% (16)	0.15	0.48	Driehuis et al. 2008b
ZEA	Grass	Netherlands	2002-2004	6% (120)	0.09	0.31	Driehuis et al. 2008a
ZEA	Grass	Netherlands	2005	13% (16)	0.13	0.21	Driehuis et al. 2008b

[1] The percentage of positive samples and total number of samples analysed.
[2] Concentration in dry matter.

As described earlier, the occurrence of ensilage-derived mycotoxins produced by *P. roqueforti* and *P. paneum*, *A. fumigatus* and *M. ruber* relates to the preservation quality of silage and depends on infiltration of oxygen during storage or during feeding-out. Very low incidences of roquefortine C and mycophenolic acid in maize and grass silages were found in a survey in the Netherlands, in which samples were analysed that were taken relatively shortly after ensiling (3 to 6 weeks) from completely sealed silages that were not yet in use for feeding purposes. Roquefortine C was detected in none of 140 maize silages and in one of 120 grass silages, and mycophenolic acid was detected neither in maize nor in grass silages (Driehuis et al. 2008a). In contrast, samples taken from opened silages at 16 Dutch dairy farms showed high incidences of roquefortine C and mycophenolic acid in surface layers of maize silages (50%) and grass silages (19%). Concentrations of both mycotoxins were highest in surface areas with visible moulds. For example, the average roquefortine C concentration in samples of visibly moulded maize silage was 16 times higher than that in silage surface samples and 270-fold higher than that in silage centre samples (Driehuis et al. 2008b). Similar observations were made in studies conducted in Germany, not only with respect to the incidence and distribution in silages of roquefortine C and mycophenolic acid but also with respect to the incidence and distribution of the *M. ruber* mycotoxins monacolin K and citrinin and the *A. fumigatus* mycotoxins gliotoxin, verruculogen and fumigaclavin C (Richter et al. 2009).

DON, zearalenone, roquefortine C and mycophenolic acid were identified as the mycotoxins with the highest incidence in a survey of mycotoxins occurring in the total diet of high-yielding dairy cows at 24 dairy farms in the Netherlands (Driehuis et al. 2008b). As expected, roquefortine C and mycophenolic acid were detected in ensiled feeds only. DON and zearalenone were detected in compound feed, feed commodities and ensiled feeds. Maize silage was found to be the most important source of all of these four mycotoxins in the diet. Maize silage represented on average 30% of the total daily feed intake of the animals, but contributed about 80% of the total dietary intake of DON and zearalenone and more than 95% of that of roquefortine C and mycophenolic acid.

Metabolism of mycotoxins in ruminants and impact on food safety

The significance of a mycotoxin occurring in feed with respect to animal health and the safety of animal food products for consumers depends on its metabolism in the animal, its toxicological effects in man and animals, and its carry-over from feed into milk, meat or organs. After intake via silage or another feed, mycotoxins, like other xenobiotics, follow the typical pharmacokinetic cascade of uptake from the gastro-intestinal tract to the blood, internal distribution, metabolism, storage/remobilization and excretion. The rumen has an important function in the metabolism of mycotoxins in ruminants. It contains a complex and dense microflora with a high biodegradative power. Some mycotoxins are rapidly metabolized in the rumen into less toxic metabolites, some are transformed into equally toxic or more toxic metabolites, while some are not transformed at all (Driehuis et al. 2010). DON and ochratoxin A are examples of mycotoxins that are transformed into less toxic metabolites in the rumen. For that reason cattle are less sensitive to these mycotoxins than non-ruminant animals such as pigs. Zearalenone is transformed in the rumen into different metabolites, with varying toxic activities. Fumonisins and aflatoxin B_1 are not metabolized in the rumen. Aflatoxin B_1 is transformed into aflatoxin M_1 in the liver of ruminants. Aflatoxin M_1 is less mutagenic and genotoxic than aflatoxin B_1, but the cytotoxicity of aflatoxin M_1 and B_1 is similar. Information concerning the metabolism of *Claviceps* and *Neotyphodium* ergot alkaloid mycotoxins and *A. fumigatus* mycotoxins is lacking. Research on the metabolism of roquefortine C and mycophenolic acid in cattle is currently in progress.

Aflatoxin B_1 is the only mycotoxin with significant carry-over into milk: between 1 and 6 percent is excreted in milk (as aflatoxin M_1) (EFSA 2004). Sixty countries now have regulations for aflatoxin M_1 in milk. The two most prevailing limit concentrations for aflatoxin M_1 in milk are 0.05 µg kg^{-1} and 0.5 µg kg^{-1} (FAO 2004). Based on a quantitative risk assessment the Codex Alimentarius established a limit concentration for aflatoxin M_1 in milk at 0.5 µg kg^{-1} (FAO 2001). Carry-over rates of DON, zearalenone, fumonisin B_1, ochratoxin A and the alkaloid ergovaline appear to be at least about 100-fold lower than that of aflatoxin B_1/M_1 (Driehuis et al. 2010). Carry-over rates of other mycotoxins frequently occurring in silage have not been assessed experimentally. However, there are no indications that significant transfer of these mycotoxins into milk occurs. More detailed information on the metabolism of mycotoxins in ruminants and their carry-over into milk can be found elsewhere (Spahr et al. 1999, Barug et al. 2006).

Prevention of mycotoxins in silage

Regarding prevention strategies, a distinction is made between field-derived and ensilage-derived mycotoxins. Prevention of field-derived mycotoxins focuses on two areas: reduction of the infection pressure of moulds and reduction of the susceptibility of the plant to fungal infections (Council for Agricultural Science and Technology 2003).

The most important prevention strategy for ensilage-derived mycotoxins is to restrict exposure of silage to oxygen. At ensiling, oxygen is entrapped in the ensiled mass, but this is rapidly consumed (within hours) by respiratory activity of the plant and (facultative) aerobic microorganisms. Once the silo is filled, the material should be protected from oxygen as quickly as possible, for instance by sealing with sheets of plastic or foil. Where appropriate, measures should be taken to prevent damage to the seal. However, since in practice the sealing of silos is never completely airtight, it is inevitable that surface layers will be exposed to air and some air will penetrate the silage during storage. A high packing density of the silage is important because it restricts air ingress during storage and after opening of the silo for feeding, when exposure to air becomes inevitable. Another factor of importance is the silage removal rate during feeding. Maintaining a high silage removal rate minimizes ingress of air into the material behind the silage face. Finally, when preventive measures have not been successful, visibly moulded silage should be discarded before feeding, since these areas are hot-spots of ensilage-derived mycotoxins, as discussed previously. More information on the impact of oxygen on silage spoilage and mould growth can be found elsewhere (Honig 1991, Pahlow et al. 2003).

In conclusion, silage can be contaminated with a variety of mycotoxins, originating from infection of the crop by moulds in the field or from growth of moulds in silage during storage or feeding-out. Prevention of mycotoxin contamination of silage requires different strategies. Field-derived mycotoxins can be reduced by application of recommended agricultural practices in crop production, whereas ensilage-derived mycotoxins can be reduced by application of adequate silage management, with emphasis on prevention of aerobic spoilage. DON and zearalenone are the mycotoxins with the highest incidence in silages and maize silage is the most important source of these mycotoxins. Based on current knowledge, aflatoxin B_1 is considered the only silage-associated mycotoxin of potential concern for the safety of milk and dairy products, due to its high carry-over rate into milk as aflatoxin M_1 and the high toxicity of this toxin. However, the incidence of aflatoxin B_1 in silages is very low in most parts of the world and national and international surveys of aflatoxin M_1 in milk indicate a high degree of compliance with existing legislation (World Health Organisation 2001, European Food Safety Authority 2004). Relatively little information is available about the effects that the ensilage-derived mycotoxins produced by *Penicillium* species and *A. fumigatus* can have on animal health and productivity. This subject should be investigated in more depth in future research.

Acknowledgements

The author acknowledges the Dutch Dairy Organisation (NZO, Zoetermeer, the Netherlands) and the Dutch Dairy Board (PZ, Zoetermeer, the Netherlands) for their financial support of parts of the research described in this paper. The author also expresses his grateful thanks to Cor van den Boogaard (FrieslandCampina, Amersfoort, the Netherlands), Margreet Hovenkamp (NZO) and Marjon Wells-Bennik (NIZO food reasearch) for their comments on the manuscript.

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