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A survey of South African silage for fungi and mycotoxins

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Samples silages, mainly maize, from dairy farms in KwaZulu Natal, South Africa were obtained and analysed for fungi and mycotoxins with a view to ascertaining if there were any dangerous levels of these contaminants present. The silage was also examined for dry matter, pH, yeasts and bacteria and these were found to vary from area to area but did not show any striking differences from other studies. Fungi were detected by a serial dilution technique using restriction agar. Several species of the mycotoxin-producing genera, namely *Aspergillus*, *Fusarium* and *Penicillium* were found. The most predominant of these was *Aspergillus fumigatus,* a known human pathogen, whereas *Fusarium* spp. were not present to any degree indicating their suppression by the ensiling process, as maize in the field is susceptible to these fungi. Mycotoxins were detected by thin layer chromatography, Vicam immuno-affinity clean-up system and high performance liquid chromatography. Four (total aflatoxins, deoxynivalenol, ochratoxin A and zearalenone) mycotoxins were found in varying quantities in the silage, while fumonisins were only found in chopped maize samples. Cytotoxicity testing was performed on human blood monocytes using mycotoxin positive and negative extracts. The results showed that cytotoxicity testing could be applied to silage extracts as a preliminary screen to detect mycotoxins. Consequently, the results indicate that maize silage can be a source of mycotoxins in the food chain, which is of particular importance where dairy cattle consume aflatoxins, due to possible contamination of milk with aflatoxin M_1.

Key words: Fungi, maize, mycotoxin, silage.

INTRODUCTION

Silage is a valuable feed adjunct that is used extensively in commercial animal farming, particularly in cattle and dairy production. Advantages of its use include its availability when normal pasture is poor or not available (Ranjit and Kung, 2000) and it is high nutrient content for the animals that consume it (Ashbell et al., 2002). Being a product from anaerobic fermentation of cut plant material, silage contains high levels of microorganisms regarded as beneficial or neutral (Danner et al., 2003). In South Africa silage is made in various ways but trench silage is a fairly common and simple method where a pit dug out of the ground, which can be up to 5 m deep. It is filled with chopped plant material, e.g., whole maize plants, which may have additives included, e.g., molasses, urea, formic acid (Donmez et al., 2003) or Lactobacillus spp. inoculum (Meeske et al., 2002). The material is compacted to remove air and is then covered with sheeting impervious to air and water, which is then held in place by weights; a convenient type being old motor tyres. The material is then left to ferment for

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several months when one end of the silo is opened and the silage is removed and transported to where the animals are being held. An alternative is to let animals feed directly from the face of the silage heap. The microorganisms usually found in well made silage are the lactic acid bacteria, which convert fermentable carbohydrates into carbon dioxide, ethanol and organic acids that lower the pH, which together with the anaerobic conditions prevents the growth of spoilage organisms such as fungi and other bacteria (Merry and Davies, 1999). Yeast can also be found in silage but the species and levels vary depending upon the conditions of ensilation in any particular batch of silage (Lowes et al., 2000).

Silage is vulnerable to contamination by spoilage moulds because ensiled materials are excellent substrates for the growth of fungi, which are ubiquitously present in the environment as spores, especially in soil in which the silage pit is enclosed (Garon et al., 2006). Fortunately, when silage is made under properly control conditions the chance of such contamination is minimised, due to the absence of oxygen, which inhibits the growth of aerobic fungi. Problems arise, however, if the silage is not made under high anaerobic conditions, e.g., due to the poor compression of the materials and leaks in the sheeting sealing the silage pit (Cavallarin et al., 2011; Woolford, 1990). When the silage clamp is opened the surface is exposed to air, allowing many fungi to grow, even if the pH is acidic, although the lactic acid content does seem to inhibit mycotoxin production (Uegaki et al., 2013). In connection with this latter point, the species (and strain) of *Lactobacillus* (that is, *L. rhamnosus*) active in the ensiling process seems to inhibit mycotoxin production, possibly because it drops the pH of the material rapidly (Dogi et al., 2013). Any fungal growth, particularly under “stress conditions” may produce mycotoxins, which are fungal secondary metabolites that are toxic towards animals and human when ingested (Pitt, 2000). Although there is evidence that some types of ensilaging may degrade some mycotoxins (Boudra and Morgavi, 2008) most mycotoxins, e.g., deoxynivalenol (DON) a *Fusarium* mycotoxin, are not removed by the ensilation process and, hence, if present will end up being ingested from silage fed animals (Baath et al., 1990; Lepom et al., 1990). Furthermore, mycotoxins (e.g., from *Aspergillus* and *Penicillium* spp.) may actually be produced in poorly made silage (Richard et al., 2007; Schneweis et al., 2000) and in addition degradation of important nutrients in the silage such as amino acids might occur (Woolford, 1990). The best way to avoid this situation is to feed the silage quickly and sometimes cattle are allowed to eat at the exposed surface of the clamp, although this may be a problem if sufficient time for fungal development is left between feedings.

There are several studies that have been done on mycotoxins in maize silage (MS) and more specifically trench silage. In general five mycotoxins: aflatoxin B_1 (AFB1) DON, fumonisin B_1 (FB1) ochratoxin A (OTA) and zearalenone (ZEA) are recognised as common contaminants of crops (Njobeh et al., 2010) being produced by the three major filamentous fungal genera, *Aspergillus*, *Fusarium* and *Penicillium*. Some other mycotoxins, however, have a more specific occurrence, e.g., patulin in fruit (Leggott and Shephard, 2000). As *Fusarium* spp. such as *F. verticillioides* are common contaminants of maize (Dutton, 2009) it would be expected that their mycotoxins (DON, FB1 and ZEA) together with AFB1, a known contaminant of maize (Brown et al., 2013) would end up in MS. All three fungal genera have been detected in silage, for example in Argentinean trench MS (Pereyra et al., 2011) with high frequencies of *Aspergillus* and *Fusarium* and lower of *Penicillium*. Interestingly, silage made in silo bags had the reverse frequency. The only mycotoxin detected was AFB1 with a range of 5.8 to 47.4 µg/kg. An earlier Argentinean study showed similar results but with a wider range of mycotoxin detected, that is, AFB1, DON, FB1 and ZEA. Studies in Mexico (Reyes-Velazques et al., 2008) and France (Garon et al., 2006) gave comparable findings with Mexican MS additionally containing OTA and the French having citrinin. With the exception of AFB1, which is highly toxic and carcinogenic and DON in the Mexican MS, none of the levels of mycotoxin found were considered present a major threat to animals consuming the silage. These results show that the contamination of silage with mycotoxin-producing genera of fungi together with the principle mycotoxins they produce is common.

As there is a paucity of information on the occurrence of fungi and mycotoxins in South African silage, an investigation was done into this aspect on silage being produced in KwaZulu Natal (KZN) mainly on MS being produced in trench silos by dairy farmers. The focus on the dairy industry was not only due to the extensive use of silage in this area but because mycotoxins such as aflatoxin can be carried over into the milk as aflatoxin M_1 (AFM1) derived from aflatoxin B_1 (AFB1) in the diet (Prandini et al., 2009) which would present an additional hazard. The samples obtained were screened for microorganisms using plate dilution methods and for mycotoxins using a thin layer chromatography/dialysis method (TLC) augmented by Vicam/fluorimetric method (Vicam) and high performance liquid chromatography (HPLC) analysis.

MATERIALS AND METHODS

All chemicals used were bought from Merck & Co. or Sigma, S.A and were of analytical grade, unless specified.

Sampling

A total number of 22 farms were sampled in the KZN province. The samples were collected both from the surface and the inside of the

Table 1. The pH and percentage dry matter of maize silage samples from three different regions of KwaZulu Natal.

Region	Mean pH	Mean dry matter (%)
Bergville (n=22)	4.4 ±0.2	20.7 ±1.3
Escort (n = 6)	3.4 ±0.1	27.4 ±1.2
Underberg (n=54)	4.7 ±0.2	31.9 ±1.0

silos. A few forage crops were also collected for analysis. A detailed description of samples is presented in Table 1. The samples were stored at -4°C until analysed.

Dry matter and pH determination

The pH and percentage dry matter (%DM) were determined by the method according to Mthiyane et al. (2001) with minor modifications. For pH determination, 125 ml of purified water was poured onto 50 g of the sample contained in a screw capped bottle. This was left for 1hr at room temperature with occasional stirring. The extract was decanted into a beaker and the pH was recorded using calibrated pH meter. The % DM was determined by drying a 40 g sample at 105°C for 96 h calculating it from the resultant final dry weight.

Fungal identification and counts

The isolation procedure for the total fungal counts was done according to the method described by Pitt and Hocking (1997). Basically 10 g of each sample were aseptically blended in 90 ml (0.1%), peptone water. A 1 ml aliquot was transferred to a test tube containing 9 ml of peptone water to make a 10^{-1} dilution. This was followed by a series of serial dilutions until a 10^{-6} dilution was reached. Aliquots of 0.1 ml of each dilution were plated onto the surfaces of Potato Dextrose agar (1% Chloramphenicol) and Di-Chlorine Rose Bengal Chloramphenicol agar (Merck, South Africa). The plates were then incubated at 25 to 32°C for five to seven days. After incubation, all the isolates were sub-cultured onto Czapek Yeast agar (CYA), Malt Extract agar (MEA) and Glucose-peptone-yeast extract agar, pH 3.7 to 4.5 (GPY). Both CYA and MEA were used filamentous fungi while GPY was used for yeasts. The filamentous fungi were identified to species level, in most cases, using the basic macroscopic and microscopic identification keys of Klich (2002) and Pitt and Hocking (1997). Yeasts were identified by their physiological characteristics using the yeast ID32C identification kit (BioM'erieux, Marcy l'Etoile, France).

Determination of mycotoxins

For the analysis of the samples for mycotoxins by thin layer chromatography (TLC) (Roberts and Patterson, 1975) the samples were aseptically blended for one minute. A 25 g sub-sample was weighed into a 250 ml flask. To the flask was added 100 ml acetonitrile: 4% (^{w}/v) aqueous potassium chloride (9:1) and the sample was homogenized by shaking for 30 min. The aqueous extract was collected by filtering through a Whatman No.1 filter paper.

The extract was partitioned twice against 100 ml trimethylpentane (iso-octane). To the aqueous extract was added 50 ml, 60% (^{w}/v) saturated sodium hydrogen carbonate, and this was partitioned three times against 50 ml dichloromethane (DCM). The DCM extract was then collected into a round-bottomed evaporating flask by passing through a bed (5-10 g) of anhydrous sodium sulphate, retaining the aqueous extract and the sodium sulphate bed. The DCM solution was evaporated *in vacuo* and the dry extract was reconstituted in a small volume of acetonitrile (2 ml). Using a sterile micropipette, the acetonitrile extract was carefully transferred into dialysis tubing (previously soaked in distilled water for 1 h) and dialysed overnight against 30% (^{v}/v) aqueous acetone. After dialysis the aqueous acetone extract was partitioned three times against 25 ml DCM through a fresh sodium sulphate bed. The DCM extract was evaporated *in vacuo*, transferred to a small vial by dissolving in a small amount of DCM, which was then evaporated under a stream of nitrogen with low heat. This constituted the neutral fraction (N).

The previously retained aqueous extract was acidified with 50 ml, 1 M H_2SO_4 and extracted with three equal volumes of 50 ml DCM. The DCM extract was passed through the previously saved sodium sulphate bed and collected and evaporated as for the N fraction. This constituted the acid fraction (A).

Fumonisin determination

For fumonisin analysis the method used to clean samples up for high performance liquid chromatography (HPLC) was that of Sydenham et al. (1992). Milled maize or blended silage samples (25 g) were mixed with 50 ml of methanol**:**water (3**:**1) in a volumetric flask, shaken for an hour and then filtered. A sample (10 ml) of the filtrate was reduced to a volume to 1 to 2 ml on a rotary evaporator at 60°C. This was then made to 10 ml with methanol:water (3**:**1,v/v). The pH of each extract was adjusted to 6.0 to 6.5 and passed through a solid phase strong anion exchange (SAX) cartridge previously conditioned by washing with 5 ml methanol and 5 ml methanol:water (3**:**1, v/v), the flow rate being maintained at 2 ml min^{-1}. Each column was then washed with 5 ml of methanol:water (3**:**1, v/v), 3 ml methanol and 5 ml methanol containing 1% acetic acid to elute the FBs. Each elute was dried under a stream of nitrogen at 60°C and stored in a refrigerator, until analysed.

For analysis by TLC samples of the extracts (20 µl from a made up volume of 200 µl extract) were spotted onto the origin of a two dimensional chromatograph plate of aluminium – backed silica fluorescent thin layer chromatography (TLC) plates (10 cm × 10 cm) (Merck Art 5554). The N and A fraction extracts were then run in DCM:ethyl acetate:propan-2-ol (90:5:5 v/v/v) in the first dimension and toluene:ethyl acetate:formic acid (6:3:1 v/v/v) in the second. The plates were air dried and viewed under UV light at 254 nm for absorbing spots and 365 nm for fluorescent spots. The fumonisin extracts were run in DCM:methanol (3:2 v/v) first dimension and butanol:water:acetic acid (12:5:3v/v/v) in the second. The plates were sprayed with p-anisaldehyde reagent (Merck, 1980), heated in the oven at 120°C for 3 min to visualize the FBs. Various spray reagents were used to confirm other mycotoxins, e.g. for ZEA, plates were sprayed with dianisidine reagent (Malaiyandi et al., 1976, modified); DON, with chromotropic acid reagent (Baxter et al., 1983) with heating at 120°C for 3 min.

For analysis by the Vicam method, analytical immuno-affinity kits were obtained from Vicam Ltd., Watertown, MA, U.S.A. for the following commonly found mycotoxins: Aflatoxins (AFs), DON, fumonisins (FBs), OTA and ZEA. Samples of commodities (30 samples silage and 6 samples chopped maize selected from those positive by TLC were extracted and cleaned up on the affinity columns as per the manufactures instructions. Quantitation was achieved by mixing the extract with appropriate developer and reading the fluorescence on a calibrated series 4 Vicam fluorimeter. Recoveries were done on by spiking maize or silage samples shown to be negative by the multi-mycotoxin screen with the appropriate mycotoxins at ppb (µg/kg) levels. Selected extracts were also examined by hplc for the commonly found mycotoxins and verification of the fluorimetry results, as follows: AFs and OTA

Table 2. Percentage of maize silage and chopped maize samples contaminated by *Aspergillus, Fusarium, Penicillium,* and other species.

Sample type	Percentage incidence			
	***Aspergillus* spp.**	***Fusarium* spp.**	***Penicillium* spp.**	**Other spp.**
Maize silage (n= 82)	70	2	19	9
Chopped maize (n = 21)	86	86	ND	ND

ND = not detected; chopped maize is the cut maize for ensilaging.

(Kokkonen et al., 2005); DON (MacDonald et al., 2004); FBs (Sydenham et al., 1992); and ZEA (Tanaka et al., 1985).

Cytotoxicity testing

Cytotoxicity testing was done to correlate the occurrence of known mycotoxins in the samples with cytotoxic response in isolated human monocytes and to ensure that no major toxic components were overlooked. All materials unless otherwise stated were obtained from Highveld Biologicals, South Africa. Blood (6 ml) was overlaid on Histopaque 1077 in a ratio of 1:1 and centrifuged for 15 min. at 3000 rpm on a bench top centrifuge at room temperature (Boyum, 1968). Monocytes were collected in the buffy coat mononuclear layer using a sterile Pasteur pipette. This was washed twice with RPMI medium by centrifugation. The cells were suspended in complete culture medium (CCM) (RMPI medium, supplemented with 10% foetal bovine serum, 1%phytohaemagglutin and 1% penicillin-streptomycin) at a rate of 5×10^5 cell/ml. These were then incubated at 37°C in a 5% CO_2 humidified incubator for 3 days, after which the cell concentration was adjusted to 5×10^5 /ml with CCM. The cell suspension was dispensed into a 96-well microplate at a rate of 100 µl per well together with 50 µl of the extract or mycotoxin standard. The standards and extracts were solubilised with 2 µl dimethylsulphoxide added to 48 µl of CCM. Suitable controls were set up with blank solubilisation medium and CCM. All experiments were done in triplicate. After a further 24 h incubation 50 µl of methyl-thiazol-diphenyltetrazolium bromide reagent (MTT) (Maenetje et al., 2008) (5 mg/ml) was added to each well and the microplates were further incubated for a further 4 h at 37°C. The resulting formazan crystals were solubilised by adding DMSO and the absorbance of each well was measured using a Benchmark plate reader at a wavelength of 540 and 620 nm. The data was statistically analysed using Sigmastat and Sigmaplot.

RESULTS AND DISCUSSION

Nearly all the pH values of the silage tested were below pH 5 and most below pH 4.5 (Table 1). There were 5 exceptions from Underberg where the pH was 7. Successful silage has to be acidic to discourage undesirable microorganism and natural development of acidic conditions indicates the presence of lactic acid bacteria responsible of the generation of short chain fatty acids (Dalie et al., 2010). An interesting point is that the silage samples from Escort were on average one pH unit lower than from the other areas, which was statistically significant ($p = <0.001$) although there does not seem to be a reason for this. From the quantity of dry matter in the silage that from the Underberg region had a higher value than the others with a mean difference as compared to the lowest from Bergville of 10% (Table 1). This is hard to explain, as it may be due to amount of cut material used in relation to its water content or growing effects related to weather.

Occurrence of Fungi in silage

Table 2 shows that fungal spores can withstand the ensiling depending upon the genus with *Aspergillus* spp. being the most resilient with only a small reduction in contamination from the chopped maize to the final MS. As expected chopped maize had a high incidence of *Fusarium* spp. with *F. verticillioides* predominating (Table 3), as this has an association with maize (Dutton, 2009). *Fusarium* spp., however, showed a marked reduction in viable propagules levels in the final MS with *Penicillium* spp. being almost absent in the starting product but appearing in the MS. Several of the species isolated (Table 3) were of importance, as *A. flavus* and *parasiticus* produce AFs and *A. carbonarius* and *ochraceus* ochratoxin. The latter species is also known to produce patulin (Cole and Cox, 1981). *Aspergillus fumigatus* was found at the highest incidence of all the fungi and this agrees with the results found by many other workers (e.g., Dos Santos et al., 2003; Richard et al., 2007). This species can produce mycotoxins, such as gliotoxin (Fox et al., 2004) but it better know as a pathogenic fungus infecting the lung. The presence of *Fusarium* spp. was severely curtailed in the silage (Table 2) and did not include *F. verticillioides* (Table 3). This is a good result, also noted by Mansfield and Kuldau (2007) as *F. verticillioides* invariably produces the mycotoxins, FBs, which have been found in MS (Eckard et al., 2011). *Penicillium* spp. was not detected in the chopped maize or the grass silage screened but were found at a moderate incidence in the MS with *P. griseofulvum* predominating. Of these *P. expansum* is a known producer of patulin, which is often found in silage (Tapia et al., 2005). It is also worth noting that *Penicillium* spp. found in silage have produce other mycotoxins, including, roquefortine C (Auerbach et al., 1998) cyclopiazonic acid (Mansfield et al., 2008) and PR toxin (Rasmussen et al., 2011). There were several other fungal species detected but not at high incidence including *Mucor* and *Rhizopus* spp. which are not recognised as mycotoxin producers. In general lower fungal propagules levels were found in the

Table 3. Incidence of fungal spp. found in maize silage.

Fungus	Incidence (%)	Mean count (cfu/g)
Aspergillus awamori	9	2×10^5
Aspergillus caespitosus	5	2×10^5
Aspergillus carbonarius	1	14×10^4
Aspergillus flavus	21	9×10^4
Aspergillus foetidus	2	7×10^4
Aspergillus fumigatus	32	9×10^3
Aspergillus japonicus	7	4×10^4
Aspergillus melleus	1	7×10^5
Aspergillus niger	11	4×10^5
Aspergillus ochraceus	4	3×10^5
Aspergillus oryzae	11	4×10^5
Aspergillus paradoxus	5	3×10^5
Aspergillus parasiticus	20	6×10^4
Aspergillus tamari	2	7×10^5
Aspergillus unguis	2	6×10^2
Fusarium graminearum	5	2×10^4
Fusarium sporotrichioides	2	8×10^4
Penicillium camemberti	1	3×10^3
Penicillium citricum	9	7×10^3
Penicillium expansum	6	8×10^3
Penicillium griseofulvum	7	6×10^6

Table 4. Yeast species isolated from maize silage.

Yeast spp. n = 82	% Incidence	Mean (cfu/g)
Candida guilliermindii	4	5×10^4
Cryptococcus humicolus	14	6×10^4
Cryptococcus laurentii	4	3×10^3
Trichosporon asahii	17	8×10^5
Trichosporon inkin	6	6×10^3
Trichosporon mucoides	10	7×10^3

interior of the silage heaps with higher ones nearer the surface indicating that the centre of the silage piles were more anaerobic than those nearer the surface, which is what would be expected when some atmospheric oxygen seeps into improperly compacted and sealed silage pits. As expected yeast species were found at moderate incidence with *Trichosporon* and *Cryptococcus* spp. predominating (Table 4). Yeasts play a role in total filamentous mould activity as they are often lactate utilisers, which encourages fungal grown but may become inhibitory when their levels exceed 10^5 and can cause reduced feed intake and disorders in the rumen (Seglar, 1999). In most of the MS samples >90%, two types of lactic acid bacteria were found, *Lactobacillus planetarium* with a mean count of 33×10^5 cfu/g and *L. buchneri* with a similar mean count of 17×10^5 cfu/g. It can be concluded, therefore that these two species mainly contribute to the lowering of pH in MS produced in KZN.

Occurrence of mycotoxins in silage

This study concentrated on the five commonly occurring mycotoxins, although the incidence of several others was screened for by TLC (Table 5). Aflatoxins (total) were the most commonly occurring mycotoxins in nearly all MS samples (97%) with a concentration range of 0.2 to 67 ppb (Table 6), which concur with the higher levels of the producing fungi *Aspergillus flavus* and *parasiticus* found in the silage (Table 3). The result also agrees with other studies: in French MS, AFB1 occurred at a range of 4 to 34 ppb (Garon et al., 2006); Mexican MS, AFB1 occurred in all samples mirroring high *Aspergillus* counts (Reyes-Velazquez et al., 2008); while Argentinean trench MS was contaminated with AFB1 at a range of 1 to 160 ppb (Pereyra et al., 2011). Cavallarin et al. (2011) found that exposure of MS to air during the ensiling process caused AFs to be formed in the end product, showing the importance of maintain anaerobic conditions to prevent this. Aflatoxins occur in a wide range of commodities (Rustom, 1997) and consequently are carefully monitored and legislated for, particularly as AFB1 is a powerful carcinogen (Lyer et al., 1994). The occurrence of this mycotoxin in silage used for dairy herds is of concern as AFB1 is converted in cattle to AFM1, which is excreted in the milk at a dilution rate with respect to levels in the feed of 1 in 180 (van Egmond, 1989). The current permitted level in milk, as legislated by South Africa and the U.S.A. is 0.05 ppm (SA Department of Health, 2004a) which is stringent (c.f. EU at 0.5 ppb) and can be difficult to detect,

Table 5. Incidence of mycotoxins using the thin layer chromatography screen in maize silage from the three areas sampled in KwaZulu Natal South Africa.

Mycotoxin	Region: Percentage occurrence of mycotoxin		
	Bergville (22)	Escort (6)	Underberg (54)
Aflatoxins	64	83	70
Deoxynivalenol	N.D.	N.D.	11
Ochratoxin A	N.D.	N.D.	N.D.
Patulin	50	N.D.	45
Zearalenone	6	N.D.	10

Table 6. Levels of mycotoxins in Underberg maize silage and fumonisin in chopped maize by Vicam and high performance liquid chromatography methods.

Mycotoxin (N = 54)	Percent incidence	Range (ppb)
Aflatoxins (Total)	97	0.2 - 67 (20% >200 ppb)
Deoxynivalenol	7	5.7-6.7
Fumonisin	ND	-
Fumonisins (Total)*	33	1.6-9.4
Ochratoxin A	7	2.6-3.4
Zearalenone	10	11.2 - 50.0

*Chopped maize (n = 21) only analysed for fumonisin by hplc; ND = none detected.

unless state of the art equipment is used. The level permitted of AFB1 in dairy cattle feed is 5 ppb (SA Department of Health, 2004b) so as to avoid the production of milk with non permitted levels of AFM1. Nearly 43% of the Bergville MS samples exceed the 5 ppb limit for dairy cattle feed (Table 6) which indicates that silage production in South Africa needs more close monitoring, if milk produced by cattle fed this silage is to meet the statuary requirements.

Two commonly found *Fusarium* mycotoxins, DON and ZEA, in maize (Marasas et al., 1977) occurred at low frequency (11 and 10% respectively) and moderate concentration (range 5.7-6.7;11.2-50 ppb respectively) in the Underberg MS in this study (Tables 5 and 6) and are generally considered not to be dangerous at these levels (Pestka and Smolinski, 2005; Zinedine et al., 2007). These results were in line with other findings; Lepom et al. (1990) found DON up to 261 ppb, in German MS and Garon et al. (2006) at a range of 100 to 213 ppb in French silage. However, these observations were not reflected in other studies; Reyes-Velazquez et al. (2008) measured DON in Mexican MS at levels above those recommended by the FDA and Eckhard et al. (2011) found DON in Swiss silage ranging from 780 to 2990 ppb with ZEA being the next highest concentration. Zearalenone was found in Iranian silage (Rashedi et al., 2012) (incidence 16.7%) and in Argentinean (range 0-350 ppb) (Gonzalez et al., 2008) and Mexican (Reyes-Velazquez et al., 2008) MS (range 168.8-482.1 ppb) at similar levels and incidence. Although *F. Verticillioides* was found in the MS and chopped maize, no FB1 was detected in any of the MS, although it was found in the starting material (chopped maize, frequency 30%, Table 6), which would indicate that it was being degraded by the ensilation process, a phenomena that has been noted by other workers (Boudra and Morgavi, 2008).

This contrast with other work, where FB1 has been detected at high levels in U.S. MS at a range of 0.2 to 10.1 ppm (mg/kg) with a frequency of 92% (Mansfield et al., 2007) and at a lower level in Mexican MS (range 100-700 ppb) (Reyes-Velazquez et al., 2008). Examination of samples using the TLC screen (Table 5) showed that OTA was absent from all samples not an unexpected result, as this mycotoxins rarely occurs in South African agricultural commodities at levels more than a few ppb (Dutton, 2003). Further analysis by HPLC showed that 7% of the Bergville samples contained lower level of OTA at around 3 ppb (Table 6). This agrees with results from Mexican MS (range 4.4-5.8 ppb) (Reyes-Velazquez *et al.,* 2008) and Slovakian MS (Mean 2.3 ppb) (Biro *et al.,* 2009).

In the TLC screen, mycotoxins other than the five commonly occurring ones, were investigated and of these patulin were found to be present at a frequency of around 45% (Table 5). Patulin- producing organisms have been isolated from silage (Tapia et al., 2005) and patulin has been reported as a problem in MS (Mansfield et al., 2008). The producing organism in the case of this silage seems to be *Penicillium expansum* and *roqueforti* both known producers (Vismer et al., 1996) whereas in other silages *Byssochlamys* spp. and its imperfect associated fungi, *Paecilomyces* spp. (Samson et al., 2009) are the

main producers (Hacking and Rosser, 1981). *Byssochalmys* spp. were not detected in these silage samples, which is fortunate as they not only produce patulin but also byssochlamic and mycophenolic acids, which also have potential toxic properties (Beuchat and Rice, 1979; Puel et al., 2005). The patulin in these samples were not quantitated further as there was no Vicam kit capable of analysing when this investigation was undertaken. It is important to note that other studies have identified other mycotoxins in MS not screened for in this study (Alonso et al., 2013) sometime at high levels. These include, citrinin (Richard et al., 2008); T-2 toxin at a dangerous level (range 179.1-249.4 ppb) (Biro et al., 2009); cyclopiazonic acid (20-1430 ppb) (Mansfield et al., 2008); fusaric acid (Shimshoni et al., 2013); roquefortine C (Driehaus et al., 2008); mycophenolic acid (Rasmussen et al., 2010); and gliotoxin, which was also at a dangerous level (up to 877 ppb) (Richard et al., 2007). Although the results reported here do not seem to indicate a reason for alarm, they do support the view that MS can be a source of mycotoxins in the food chain and that a much wider screening process (Rasmussen et al., 2010) for all mycotoxins posing a potential threat to animals and the food chain should be routinely applied where possible.

Human monocytes exposed to several sample extracts were examined by the MTT cytotoxicity assay to see if there were any toxic principles present in the silage other than the mycotoxins screened for. The control sample was that of a neutral silage extract (dialysis method) which had not shown any evidence of mycotoxin presence. The undiluted extract as compared to the control showed an inhibition of 5% which is negligible compared to extracts with known mycotoxins present. The effects of an extract shown to be positive for aflatoxin was also tested and a clear inhibitory effect was shown with the highest been evoked by the undiluted extract at 55% inhibition as compared to the untreated control. This result is encouraging in terms of using cytotoxicity testing, as a general screen for toxins in silage, which would not only indicate the presence of mycotoxins but other compounds such as pesticides. Rasmussen et al. (2011) also showed the value of cell cultures in assessing the toxicity of silage and isolated fungi but this methodology used a Caco-2 cell line requiring a cell culture facility, unlike the cells used here, which were isolated human monocytes, which may be substituted with animal, e.g. pig, if necessary (Mwanza et al., 2009).

Conclusion

The main objective of this study was to examine MS produced in dairy farming areas of KZN to see what dangerous mycotoxins may be present, if any, and cause concerns for milk production and milk quality. In general the silage was of good quality and, although mycotoxins could be detected, in the main they were at levels not considered to be dangerous. The possible exception to this is that levels of AFB1 were found which were in excess of permitted legislated levels, although these were unlikely to produce bulk milk with AFM1 in the milk over the legislated level of 0.05 ppb. With a few specific examples of high concentrations of certain mycotoxins found by other workers, these results agree with those reported in the literature. The results, however, should not lull us into a false sense of security, not only in light of the dangerous levels of mycotoxins found in MS by other worker mentioned above but because recent legislation in South Africa has added various mycotoxins to the list of those specifically controlled in farm feeds, namely DON, FB1, OTA, trichothecene, moniliformin, mycophenolic acid, ZEA, nivalenol, patulin, and citrinin (SA Department of Health, 2004b).

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