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ORIGINAL ARTICLE

Gliotoxin contamination in and pre- and postfermented corn, sorghum and wet brewer's grains silage in Sao Paulo and Rio de Janeiro State, Brazil

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Keywords
Aspergillus fumigatus and related species, fungi, mycotoxin production, silage.

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Abstract

Aims: The aim of this study was to determine total fungal counts and the relative density of *Aspergillus fumigatus* and related species in silage samples intended for bovines before and after fermentation as well as to monitor the natural occurrence of gliotoxin in silage samples (pre- and postfermentation).
Methods and methods: The survey was performed in farms located in São Paulo and Rio de Janeiro States in Brazil. In addition, the ability of *A. fumigatus* strains and related species strains to produce gliotoxin was also evaluated. A total of 300 samples were taken, immediately after opening of the silo (3–5 months) and during the ensiling period. Fungal counts were done by the surface-spread method. Gliotoxin production ability of isolates and natural contamination were determined by HPLC.
Results: All postfermented samples had a total number of moulds exceeding 1×10^4 CFU g^{-1}, with *Aspergillus* sp. as the most prevalent genus. Frequency of strains, among *A. fumigatus* and related species, was able to produce gliotoxin was similar in pre- and postfermented samples, except for sorghum, which showed differences between both kinds of samples. The highest toxin levels were produced by strains isolated from postfermented samples. More than 50% of the samples showed gliotoxin contamination levels that exceeded concentrations known to induce immunosuppressive and apoptotic effects in cells.
Conclusions: The present data suggest that care should be taken because gliotoxin contamination in feedstuffs could affect productivity and also present a health risk for herds.
Significance and Impact of the Study: Gliotoxin was found at quite important concentrations levels in pre- and postfermented substrates and its presence could therefore probably affect the productivity and health of herds. Current conservation and management practices do not avoid contamination with *A. fumigatus* on silage. Therefore, farm workers should be adequately protected during its handling.

Introduction

Currently, Brazil is the world's main producer of beef, possessing the largest commercial herd in the world (accounting for 15% of total production). The fodder-based feeding of the herd is one aspect that has aroused interest of importing countries, so that exports have grown significantly in recent years. Corn (*Zea mays* L.) and *sorghum* (*Sorghum bicolor* L. Moench) are the most widely grown crops in the Americas and are extensively used for animal feeding because of their nutritional values. In Brazil, about 89% of the total corn and sorghum production is destined to animal feeding (Rodrigues *et al.* 2002a,b). The wet brewer grains, brewery industry residues, which have a good nutritional value and a low-cost supply, are widely used to feed cattle in the State of Rio de Janeiro. The production of silage entails incorporation of the processed materials, and their storage is based on the principle of preservation under anaerobic conditions. The growth of lactic acid bacteria promotes a natural fermentation that lowers the pH to a level at which clostridial and most moulds growth is inhibited (Leonel *et al.* 2008). Temperature, humidity, oxygen availability and pH conditions vary during the silage process, and microbiota may also change from one stage to another. Nutritional value of ensiled forages is similar to that of fresh forages (Leonel *et al.* 2008). However, poor storage conditions – including excessive moisture or dryness, condensation, heating, leakage of rainwater and insect infestation – can lead to undesirable mould contamination, mycotoxin production and the reduction in nutritional value. The fungal growth reduces the nutritional value and may result in mycotoxins production and allergenic spores that constitute a risk factor for both human and animal health (Nout *et al.* 1993; Boysen *et al.* 2000; Cleveland *et al.* 2003; dos Santos *et al.* 2003; Frisvad *et al.* 2006; Richard *et al.* 2009).

Mycotoxins are low-molecular-weight products of the fungal secondary metabolism, produced mainly by *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* genera. These metabolites are toxic to humans and animals when consumed or inhaled, and exposure to mycotoxins through contaminated feed is one of the major risk factor to ruminant health (Bennett and Klich 2003). Thermophilic *Aspergillus fumigatus* is a mould species in silage, and many strains can produce several mycotoxins that may cause health problems in dairy cattle (Dutkiewicz *et al.* 1989; dos Santos *et al.* 2003; Pereyra *et al.* 2008). Mycotoxins produced by *A. fumigatus* include tremorgens (Land *et al.* 1993), clavine alkaloids that appear to be responsible for abortions (Moreau 1979) and gliotoxin. This toxin has been reported as the main and most potent of group because it can affect immune system (Belkacemi *et al.* 1999; Yamada *et al.* 2000; dos Santos *et al.* 2003; Pereyra *et al.* 2008). In fact, numerous syndromes in ruminants are likely to be produced by fungal or toxin ingestion present in spoiled silage (Seglar 1999).

Kwon-Chung and Sugui (2009) analysed the role of gliotoxin in the pathobiology of *Aspergillus fumigatus*. This mycotoxin has been suspected as one of the most likely virulence determinants among various secondary metabolites produced by the species. Gliotoxin is a dipeptide characterized by the presence of a disulphide bridge across the piperazine ring. The disulphide bridge allows the cross-linking with cysteine residues in proteins and generates deleterious reactive oxygen species (ROS) through a redox cycle between the reduced and oxidized forms. This mechanism of ROS generation is believed to be responsible for the toxicity of gliotoxin (Gardiner *et al.* 2005). A survey of patients of a cancer centre in the United States reported a frequency of gliotoxin production of 93% among clinical *A. fumigatus* isolates (Lewis *et al.* 2005). These results support the hypothesis that gliotoxin production might act *in vivo* as a virulence factor required to establish *A. fumigatus* infection.

The aims of this study were to determine total fungal counts and the relative density of *A. fumigatus* and related species in silage samples, as well as to find out the natural occurrence of gliotoxin in silage samples intended for beef cattle feed (pre- and postfermented). Moreover, the ability of strains of *A. fumigatus* and related species to produce gliotoxin was evaluated.

Materials and methods

Sample collection

A total of 232 samples of corn silages (58 corn silos), 48 samples of sorghum silages (12 silos) and 20 samples of wet brewer grains silages (five silos), in the period from June 2007 to October 2007 and February 2008 to May 2008, were collected. These silos were located in São Paulo and Rio de Janeiro states. Samples were taken before (prefermented) and 90 days after compacting of the silo (postfermented). To guarantee a correct sampling, each silo was imaginary divided along its length into three equal parts with four sections each: upper, lower, border and middle sections. Six subsamples (500 g) were collected from each section to obtain a total of three kilograms sample, which was immediately homogenized, quartered and tested for moisture content, fungal isolation frequency and relative density. These render a total of twelve laboratory samples per silo. Samples were also immediately tested for dry matter content (DM %), pH and water activity (a_W). Then, they were stored at 4°C until gliotoxin analysis.

Physical evaluation of the samples

Each laboratory sample (100 g) was dried in a forced air oven for 72 h at 65°C. Duplicated samples were weighed, and the percentage of dry matter was determined (Ohyama *et al.* 1975). Evaluation of pH was performed as follows: Samples (30 g) were placed into beakers, completely covered with distilled water and left in contact for 30 min before pH determination. The measurements were performed using pH indicator strips obtained from Merck chemical co. (Merck KGa, Darmstadt, Germany).

Mycological survey

Total fungal counts of samples were performed onto three different culture media: dichloran rose bengal chloramphenicol agar (DRBC), a general medium used for estimating total culturable mycobiota (Abarca *et al.* 1994), and dichloran 18% glycerol agar (DG18), a low a_W medium that favours xerophilic fungi development (Pitt and Hocking 1997). Quantitative enumeration was carried out using the surface-spread method. Ten grams of each sample was homogenized in 90 ml of 0·1% peptone water solution for 30 min in an orbital shaker. Serial dilutions (10^{-2} to 10^{-3}) were made, and 0·1 ml aliquots were inoculated in duplicates onto the culture media. Plates were incubated at 25°C for 7–10 days in darkness.

Colonies representative of *Aspergillus fumigatus* (uniseriate aspergilli, columnar conidial heads in shades of green and flask shaped vesicles) were called *A. fumigatus* and related species.

The results were expressed as isolation frequency (% of samples in which each genera was present) and relative density (% of isolation of *A. fumigatus* and related species among strains of the same genera).

Gliotoxin production

All *A. fumigatus* and related species strains were preserved on agar slants Malt Extract Agar (MEA) at 4°C until assay for gliotoxin production. These strains were grown on yeast extract liquid medium (YES) (sucrose 40 g, yeast extract 20 g and distilled water to 1000 ml). YES was sterilized by autoclaving for 20 min at 121°C. Erlenmeyer flasks (250 ml) with 100 ml of YES each were inoculated with an agar plug of each strain (5 mm diameter) from MEA cultures, incubated for 7 days at 28°C and for 2 days at 37°C in shaking at 1400 rev min^{-1}.

Extraction and detection of gliotoxin

Yeast Extract Sucrose broths were filtered through Whatman Nº 1 filter paper. The filtrate was extracted twice by shaking for 10 min with 50 ml of chloroform at 25°C. The chloroform fractions were pooled and evaporated to dryness on a rotary evaporator. Dried extracts were stored at −70°C and resuspended in 200 μl of mobile phase prior to gliotoxin analysis by HPLC.

Detection and quantification of gliotoxin

Gliotoxin was determined following the methodology proposed by Pena *et al.* (2010). The HPLC apparatus used for gliotoxin determination was a Perkin Elmer 200 Series HPLC System equipped with an autosampler and UV detection. Briefly, gliotoxin separation was performed at room temperature on a Phenomenex Luna RP C18(2) column (150 × 4·6 mm, 5 μm; Phenomenex Inc., Torrance, CA, USA) fitted with a C18 guard column using an isocratic mode: 75% (1% acetic acid in water) and 25% acetonitrile. A column washing of 5 min at 95% of acetonitrile followed by 5 min of stabilization at the running conditions was performed between chromatographic runs. The retention time, which was slightly dependent on sample matrix composition, was properly checked by co-injection technique, as shown in Figs 1 and 2 for samples from the gliotoxin-producing ability assays and naturally contaminated samples, respectively. Detection was performed at 254 nm. The standard solutions in mobile phase were prepared from a 5 mg ml^{-1} solution of pure gliotoxin (Sigma-Aldrich) in chloroform, after solvent evaporation. The detection limit determined as a rate s/n = 3 was 0·2 μg g^{-1}, and the limit of quantification, as a rate s/n = 10, was 0·9 μg g^{-1}, where s means signal (intensity of the toxin peak) and n means signal noise.

Thermotolerance determination

Growth of *A. fumigatus* and related species was determined on MEA and CZA according to Hong *et al.* (2005). Conidia from a 7- to 14-day incubation were mixed into semisolid medium (0·2% agar and 0·05%, Tween 80) and inoculated in Petri dishes. The plates were incubated in the dark at 10 and 55°C with intervals of 5 C, and growth was observed.

Gliotoxin extraction in silages of corn, sorghum and wet brewer grains

Gliotoxin incidence was determined following the methodology proposed by Boudra and Morgavi (2005). Samples were dried at 48°C for 72 h in a forced air oven. Distilled water (10 ml) was added to flasks containing 10 g of sample followed by addition of 40 ml dichloromethane. The sample–solvent mixture was soaked at room temperature for 2 h, followed by 15 min of mechanical stirring, and

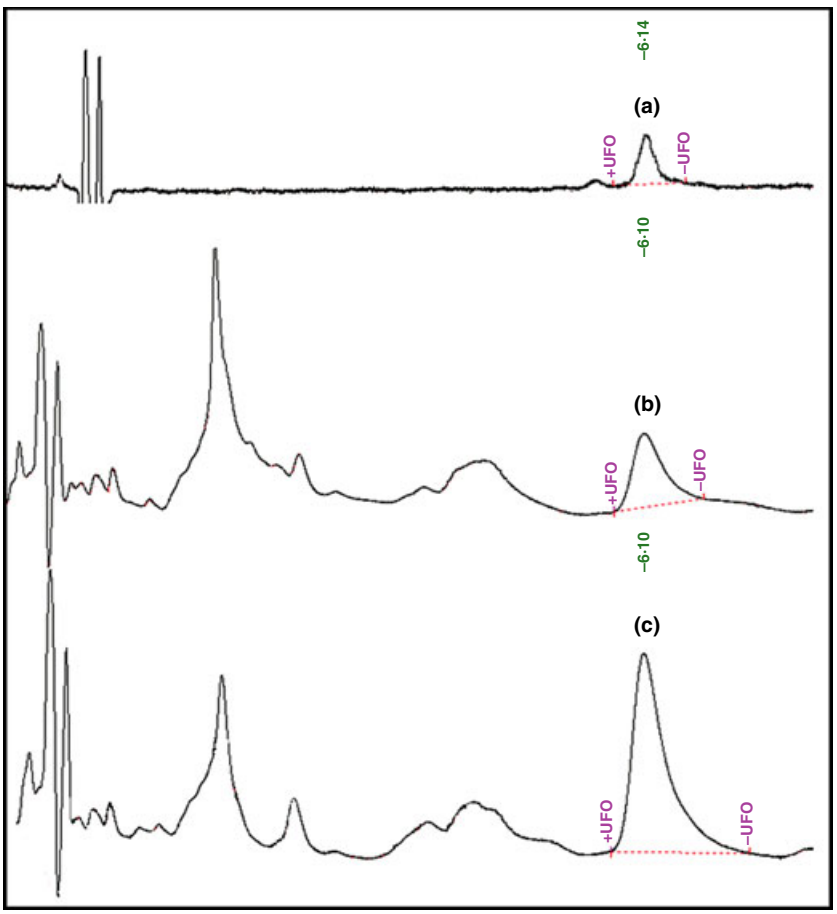

Figure 1 (a) Chromatogram of Gliotoxina standard of 2 ppm (t_R 6,10 min, Volume Injected 10 μl, Area = 10240); (b) Chromatographic profile of typical of a toxigenic capacity sample (t_R 6,00 min, 2.4 ppm, Injected Volume 20 μl. Area 5780). (c) Gliotoxin enriched extract (c.a. 4.4 ppm, Area 16020).

filtered through filter paper (Whatman Inc., Clifton, NJ, USA). Three millilitre of the filtrate extract was evaporated to dryness under gentle N_2 flow. The residue was redissolved in 500 μl of mobile phase for gliotoxin analysis.

Statistical analyses

Data analyses were performed by analysis of variance (ANOVA). Data were transformed using the logarithmical function $\log_{10}(x + 1)$ before applying ANOVA. Duncan's test was used for comparing CFU g^{-1} total fungal counts on different culture media, and Fisher's protected LSD test was used for comparing means of gliotoxin contamination and physical evaluation of samples data (Quinn and Keough 2002). The analyses were conducted using PROC GLM in SAS (SAS Institute, Cary, NC, USA).

Results

Physical evaluation of the samples

Physical properties of samples revealed considerable differences in both moisture content (a_W) and pH between individual samples. Water activity levels in samples ranged between 0·624 ± 0·090 and 0·976 ± 0·010. The pH values varied from 4·2 to 6·5 values. Mean DM % of samples was 39·7% varying in a range from 38 to 42%. No statistically significant differences were observed when comparing physical characteristics of pre- and postfermented samples.

Mycological survey

Table 1 describes total fungal counts onto DRBC and DG18 media present in pre- and postfermented samples during the sampling period. Total fungal counts of all samples obtained from DRBC agar, and counts of xerophilic fungi obtained from DG18 agar showed a high degree of contamination with counts over 1×10^3 CFU g^{-1}. Fungal counts were significantly higher in comparison with pre- and postfermented samples ($P < 0{\cdot}0001$, $F = 113{\cdot}3$). The LSD test revealed significant differences among counts from pre- and postfermented samples. On the other hand, no significant differences were observed between sections in a silo ($P > 0{\cdot}05$). The same was observed when comparing DRBC and DG18 culture media.

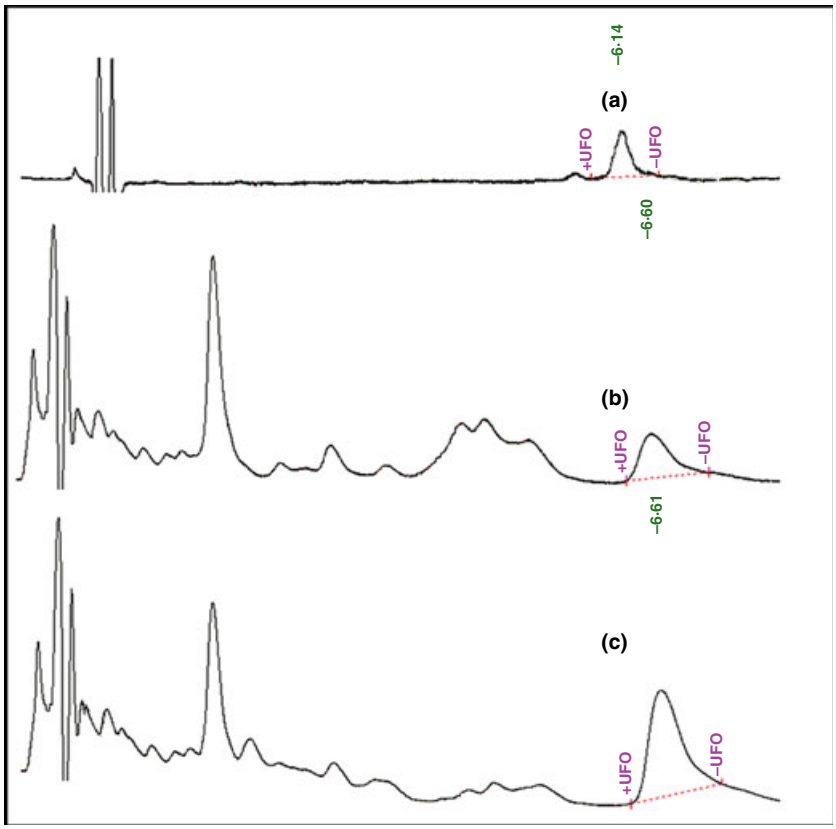

Figure 2 (a) Chromatogram of Gliotoxina standard of 2 ppm (t_R 6,10 min, Volume Injected 10 μl, Area = 10240); (b) Chromatographic profile of typical sample (t_R 6,61 min, 2.4 ppm, Injected Volume 20 μl. Area 12363). (c) – Gliotoxin enriched extract (c.a. 4.4 ppm, Area 22603).

Table 1 Fungal counts (CFU g^{-1}) from several type silages in DRBC and DG18 culture media

		Fungal counts (CFU g^{-1})* (media ± SD)				
			Silage section			
Samples	Silage	Culture media	Upper	Middle	Borders	Lower
Corn	Prefermented	DRBC	$3.7 \times 10^3 \pm 3.3 \times 10^3$	$3.5 \times 10^3 \pm 5.6 \times 10^3$	$3.8 \times 10^3 \pm 6 \times 10^3$	$2.2 \times 10^3 \pm 3.4 \times 10^3$
		DG18	$2.0 \times 10^3 \pm 1.9 \times 10^3$	$1.8 \times 10^3 \pm 2.9 \times 10^3$	$2.4 \times 10^3 \pm 4.4 \times 10^3$	$2.7 \times 10^3 \pm 3.9 \times 10^5$
	Postfermented	DRBC	$1.3 \times 10^5 \pm 9.4 \times 10^4$	$1.4 \times 10^5 \pm 2.6 \times 10^5$	$6.0 \times 10^4 \pm 1.1 \times 10^5$	$5.6 \times 10^4 \pm 1.3 \times 10^5$
		DG18	$3.1 \times 10^4 \pm 6.3 \times 10^4$	$1.7 \times 10^4 \pm 2.6 \times 10^4$	$2.1 \times 10^4 \pm 4.0 \times 10^4$	$2.5 \times 10^4 \pm 6.7 \times 10^4$
Sorghum	Prefermented	DRBC	$5.1 \times 10^3 \pm 7.0 \times 10^3$	$1.5 \times 10^4 \pm 1.2 \times 10^4$	$2.3 \times 10^4 \pm 4.5 \times 10^4$	$1.3 \times 10^4 \pm 2.6 \times 10^4$
		DG18	$2.9 \times 10^3 \pm 3.5 \times 10^3$	$2.1 \times 10^3 \pm 2.2 \times 10^3$	$2.9 \times 10^3 \pm 5.2 \times 10^3$	$3.4 \times 10^3 \pm 3.2 \times 10^3$
	Postfermented	DRBC	$2.4 \times 10^4 \pm 2.2 \times 10^4$	$5.4 \times 10^4 \pm 6.8 \times 10^4$	$4.9 \times 10^4 \pm 7.6 \times 10^4$	$2.7 \times 10^4 \pm 2.6 \times 10^4$
		DG18	$1.29 \times 10^4 \pm 1.1 \times 10^4$	$1.5 \times 10^4 \pm 1.6 \times 10^4$	$2.2 \times 10^4 \pm 2.9 \times 10^4$	$5.2 \times 10^4 \pm 9.9 \times 10^4$
Wet brewer grains	Prefermented	DRBC	$2.8 \times 10^3 \pm 2.1 \times 10^3$	$1.8 \times 10^3 \pm 8.3 \times 10^2$	$2.5 \times 10^3 \pm 1.8 \times 10^3$	$2.2 \times 10^3 \pm 1.9 \times 10^3$
		DG18	$1.6 \times 10^3 \pm 1.9 \times 10^3$	$1.8 \times 10^3 \pm 2.3 \times 10^5$	$1.5 \times 10^3 \pm 1.8 \times 10^3$	$1.8 \times 10^3 \pm 1.5 \times 10^3$
	Postfermented	DRBC	$1.3 \times 10^5 \pm 1.1 \times 10^5$	$2.3 \times 10^5 \pm 2.3 \times 10^3$	$1.8 \times 10^5 \pm 4.4 \times 10^5$	$2.5 \times 10^4 \pm 1.9 \times 10^4$
		DG18	$1.5 \times 10^5 \pm 2 \times 10^5$	$5.2 \times 10^4 \pm 5.6 \times 10^4$	$6.2 \times 10^3 \pm 6.8 \times 10^3$	$2.3 \times 10^4 \pm 2.5 \times 10^4$

DRBC, dichloran rose bengal chloramphenicol; DG18, dichloran glycerol 18%.
*SD, Standard deviation. Detection limit: 1×10^2 CFU g^{-1}. Maximum recommended level: 1×10^4 CFU g^{-1} (GMP, 2006).

Table 2 shows the isolation frequency of *Aspergillus* spp. (%) and relative density of *A. fumigatus* and related species (%). As can be observed, the most prevalent isolated genus was *Aspergillus*, while *A. fumigatus* and related species relative density was higher in postfermented silo samples than in prefermented ones.

Table 2 Isolation frequency of *Aspergillus* spp. (%) and relative density of *Aspergillus fumigatus* and related species (%)

		Aspergillus spp.		*A. fumigatus* and related species	
Samples	Silage	Number of strains/total	Isolation percentage (%)	Number of strains/total	Relative density (%)
Corn	Prefermented	50/134	37	13/50	26
	Postfermented	209/384	54	67/209	32
Sorghum	Prefermented	8/34	23·5	9/34	26
	Postfermented	42/84	50	18/42	43
Wet brewer grains	Prefermented	4/22	18	1/4	25
	Postfermented	9/40	22·5	2/9	22

Table 3 Gliotoxin production by strains of *Aspergillus fumigatus* and related species and natural levels in several types of silages

		Gliotoxin production by strains (mg l^{-1})*			Gliotoxin natural levels (mg kg^{-1})*	
Samples		Positive strains†	(%)	Level range	Contamination frequency (%)	Level range
Corn	Prefermented	8/13	61·50	d-5	20	d-2
	Postfermented	42/67	65	d-60	70	d-34
Sorghum	Prefermented	5/9	55·50	d-3	30	d-2
	Postfermented	15/18	83	d-34	60	d-23
Wet brewer grains	Prefermented	1/1	100	d-3	30	d-10
	Postfermented	2/2	100	d-46	50	d-22

*LOD: 0·2 μg g^{-1}, LOQ: 0·9 μg g^{-1}, d, detectable.
†Number of strains.

Gliotoxin production and thermotolerance determination

Frequency of strains able to produce gliotoxin was similar in pre- and postfermented samples, except for sorghum that showed differences between both kinds of samples. Gliotoxin levels produced by strains isolated from corn samples varied between 0·1 and 60 mg l^{-1}, whereas those obtained from strains isolated from sorghum and wet brewer's grains samples ranged from 0·1 to 34 mg l^{-1} and from 0·1 to 46 mg l^{-1}, respectively. The strains isolated from corn sorghum and brewer postfermented samples produced in general higher levels of gliotoxin than those isolated from prefermented samples Table 3.

All strains among *A. fumigatus* and related species that were able to produce gliotoxin were also able to grow at 50°C, but did not grow at 10°C on MEA and CZA. These strains had rapid-growing velutinous colonies, subclavate vesicles and abundant conidiation.

Gliotoxin incidence analysis

The natural incidence of gliotoxin (mg kg^{-1}) from silage samples was determined, and results are shown in Table 3. Gliotoxin levels from different analysed substrates were significantly different ($P < 0{\cdot}0001$, $F = 313{\cdot}67$). Other significant differences were observed between pre- and postfermented samples ($P < 0{\cdot}05$, $F = 3{\cdot}26$) and between different sections of the silos ($P < 0{\cdot}05$, $F = 4{\cdot}87$).

Discussion

The major objective of this study was to determine the gliotoxin incidence in samples obtained from corn, sorghum and wet brewer grains silages. Even though variations in a_W levels were observed, they still remain in the range that allows the fungal growth. The silos were cut by shovelling for animal feeding. This practice breaks the compaction of the silo content and favours aeration, allowing the development of yeasts and moulds. It was observed that the permeability of plastic sheeting used to seal the silo allowed penetration of some rainfall.

All fungal counts from postfermented samples exceeded 1×10^4 CFU g^{-1}, the recommended limits to ensure hygienic quality (Good Manufacturing Practices, GMP 2006), showing a high fungal activity. These results agree with those reported by dos Santos *et al.* (2003) who found CFU counts over 10^4 CFU in 80% of silage samples and González Pereyra *et al.* (2008) who found over 10^6 CFU in 75% of corn silage samples from Argentina. These findings reveal a similar occurrence pattern in South America. Mor and Singh (2000) and El-Shanawany *et al.* (2005) found levels between 10^3 and 10^5 CFU from silage in Egypt and India. The examination of the samples

for the presence of fungi indicated that *Aspergillus* was the most prevalent genus, followed by *Penicillium* spp. Similar results have been reported in grains, silage and animal feed samples (Dalcero *et al.* 1997; Schneweis *et al.* 2000; Oude Elferink *et al.* 2001; dos Santos *et al.* 2003; Rosa *et al.* 2006; Keller *et al.* 2007; Pereyra *et al.* 2008). The microaerophilic conditions and moderately low pH inside the silo inhibit the development of some fungi strains and favour others which tolerate these environmental conditions, such as *A. fumigatus*.

The most common species of *Aspergillus* that cause invasive aspergillosis worldwide are *A. fumigatus*, *A. terreus*, *A. flavus* and *A. niger*. To correlate the pathogenic potential of *A. fumigatus* with the ability to produce gliotoxin and to investigate the taxonomic distribution of gliotoxin-producing *Aspergillus* strains among clinical isolates, Kupfahl *et al.* (2008) studied a total of 158 *Aspergillus* isolates comprising four different species *A. fumigatus* (100), *A. terreus* (27), *A. niger* (16) and *A. flavus* (15) collected from different medical centres (some isolated from probable cases of aspergillosis) and from environmental samples. Gliotoxin was detected in most culture filtrates of *A. fumigatus* of both clinical (98%) and environmental (96%) origin. The toxin was also detected, with decreasing frequency, in culture filtrates of *A. niger* (56%), *A. terreus* (37%) and *A. flavus* (13%). The higher gliotoxin concentrations were detected in *A. fumigatus* strains cultures, whereas the gliotoxin productivities of other *Aspergillus* species were significantly lower. Given these findings, only *A. fumigatus* and related species strains were selected in this work to determine the gliotoxin-producing ability and its relation with gliotoxin contamination in silages.

Traditionally, the identification of *A. fumigatus* was performed using the colony patterns and the morphology of the conidiogenous structure and conidia. Hong *et al.* (2005) examined the variability within *A. fumigatus* Fresenius and related species using macro-morphology, micromorphology, growth temperature regimes and extrolite patterns. In addition, DNA analyses including partial β-tubulin, calmodulin and actin gene sequences were used. They concluded that DNA analyses separated strains, considered as *A. fumigatus* before, into four groups including *A. fumigatus* sensu stricto, *A. lentulus*, *A. fumigatiaffinis* and *A. novofumigat*us. However, based on some morphological characteristics, they observed that (sub) globose vesicles in *A. fumigatus* sensu stricto were rare, but common in the other species. In the same way, all strains of *A. fumigatus* sensu stricto did not grow at 10°C, but grew at 50°C. Moreover, the comparison of the profiles of extrolites produced by *A. fumigatus*, *A. lentulus* and related species demonstrated that *A. fumigatus* sensu stricto strains were the only strains able to produce gliotoxin. In this study, although the sequence analysis has not been carried out, the strains among *A. fumigatus* and related species that produced gliotoxin and grow at 50°C showed strong morphological resemblance to *A. fumigatus* sensu stricto.

In this work, a high percentage of strains among *A. fumigatus* and related species isolated from pre- and postfermented samples were able to produce gliotoxin. Also, the higher levels were produced by strains from postfermented samples. These results suggest that the environmental conditions prevalent in postfermented silage (high temperature, low oxygen tension, acidic pH) could favour the gliotoxin production. There are many studies about the influence of ecophysiological factors on growth and mycotoxin production in *Aspergillus* spp. However, no information is available about the influence of these parameters on growth and gliotoxin production by *A. fumigatus* strains. Kosalec *et al.* (2005) studied the *in vitro* production of gliotoxin in clinical strains of *A. fumigatus* and demonstrated that incubation at 37°C resulted in higher gliotoxin production than at 25°C. They found that gliotoxin was produced during mycelial growth. Thus, faster growth at higher temperatures was associated with greater gliotoxin production. In the same way, Hall and Denning (1994) studied the growth of 24 clinical *Aspergillus* isolates at low oxygen tensions including *A. fumigatus* (10), *A. terreus* (6), *A. niger* (6), *A. nidulans* (1) and *A. flavus* (1) strains and reported that 21 of them were able to grow at low oxygen tensions. Probably, the gliotoxin could be produced at the same conditions. Boudra and Morgavi (2005) evaluated the capacity of 14 *A. fumigatus* strains to produce gliotoxin. After 3 days of incubation, this toxin was found at levels lower than those reported here. In addition, they determined the effect of culture conditions on biomass and gliotoxin production by an *A. fumigatus.* In contrast to the findings of this work, the acid medium (pH 4) reduced the synthesis of gliotoxin. However, it is important to stress that neither the used medium reflects the silage substrate nor the used *A. fumigatus* strain was native from silage. The strain used in the research was isolated from sea sediment and therefore could not be adapted to silo conditions.

Previous studies claimed that environmental isolates of *A. fumigatus* rarely produce gliotoxin, in contrast to clinical isolates (Lewis *et al.* 2005). However, in the present work, a high percentage of strains from pre- and postfermented samples, considered as environmental strains, were able to produce gliotoxin. In agreement with our results, other studies showed the isolation of *A. fumigatus* from cereal grains and other animal feed silages with ability to produce gliotoxin (dos Santos *et al.* 2003; Garon *et al.* 2006; González Pereyra *et al.* 2008; Kupfahl *et al.* 2008; Pereyra *et al.* 2008; Richard *et al.* 2009).

The present survey shows that more than 50% of samples were contaminated with gliotoxin. High gliotoxin frequencies were also reported in corn silage and ready feed intended for dairy cattle from Argentina (Pereyra *et al.* 2008).

Recently, Scharf *et al.* (2012) summarized the recent progress in elucidating the gliotoxin biosynthetic pathway and its role in virulence. They stated that the toxic and immunosuppressive characteristics of gliotoxin towards the host's immune effector cells implied a substantial role of this compound in fungal pathogenicity. Accordingly, gliotoxin was shown to be produced during the infection process and was detected in lungs and sera of mice and also in humans infected with *A. fumigatus*. It is important to emphasize that much of the contamination comes from prefermented substrate because of the excessive manipulation in the field during the preparation of the silos, which will increase fungal counts in the substrates. These results are very important because cellular studies have shown that even low levels of gliotoxin might cause harm to animals. In fact, Upperman *et al.* (2003) and Watanabe *et al.* (2003) observed that low concentrations ($0{\cdot}01\ \mu g\ ml^{-1}$) of gliotoxin could induce immunosuppressive and apoptotic effects in cells. The concentrations of gliotoxin reported here far exceed this harmful value and therefore suppose a real risk for animals.

In conclusion, *A. fumigatus* was found at a high density in different raw materials commonly used for feed intended for Brazilian beef cattle. Simultaneously, gliotoxin was found at quite important concentrations levels in pre- and postfermented substrates, and therefore, its presence could probably affect productivity and health of herds. Because of current conservation and management practices, it is not possible to avoid the contamination with *A. fumigatus* on silage. Therefore, the farm workers should be adequately protected during handling. Future control strategies must be implemented to prevent the growth of gliotoxin-producing strains and the consequent production of their toxic metabolites.

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