FUNGI AND AFLATOXIN B_1 IN PRE AND POST-FERMENTED SORGHUM TRENCH TYPE SILOS DESTINED TO BOVINE INTENSIVE-REARING IN BRAZIL

INCIDENCIA DE HONGOS Y AFLATOXINA B_1 EN SILOS DE SORGO TIPO TRINCHERA PRE Y POST-FERMENTADOS DESTINADOS A LA CRÍA INTENSIVA DE BOVINOS EN BRASIL

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Abstract

The harmful effects of fungi in sorghum silage are an important matter in many countries with hot climates. This culture is very susceptible to fungal contamination and both the storage and processing of this substrate are ideal for the development of fungi there are able to produce mycotoxins. The contamination with mycotoxins, secondary products of the metabolism of certain fungi, that promotes risks to both animal and human health, causes numerous losses for the country. The aim of this study was to identify the toxicogenic fungi present in sorghum silage for beef cattle consumption and determine the occurrence of aflatoxin B_1 (AFB_1) in the same substrate. A total of 60 pre-fermented and 60 post-fermented samples of sorghum silage were analyzed. Total fungal counts and natural incidence of toxigenic *Aspergillus, Penicillium* y *Fusarium* species were performed on dichloran rose bengal chloranphenicol agar, dichloran glycerol agar 18 % and Nash-Snyder culture media. Aflatoxin B_1 contamination was determined using high pressure liquid chromatography (HPLC). About 30 % of samples from pre-fermented sorghum and 55 % of post-fermented samples were above the recommended limits (1.0×10^4 CFU g^{-1}). The most frequent fungal species in both types of sorghum samples were *A. flavus, P. citrinum, P. islandicum* and *F. verticillioides.* An average of 32 % samples, pre and post-fermented, were positive for AFB_1. The presence of fungi and AFB_1 in the feedstuffs indicates contamination. This toxin could affect animal

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productivity and health. This fact requires periodic monitoring to prevent the occurrence of mycotoxicosis in animal production.

Key words: aflatoxin B_1, bovine intensive-rearing, fungi, silage.

Resumen

Los efectos nocivos de la contaminación fúngica en ensilaje de sorgo son de importancia en países de clima cálido. Las condiciones de almacenamiento y procesamiento de este sustrato son ideales para el desarrollo de hongos capaces de producir toxinas. La presencia de micotoxinas, productos del metabolismo secundario de ciertos hongos, causan numerosas pérdidas económicas en el país y promueven riesgos para la salud humana y animal. El objetivo de este estudio fue identificar hongos toxicogénicos en ensilaje de sorgo destinado a la cría intensiva de ganado bovino y determinar la presencia de aflatoxina B_1 (AFB_1) en el mismo. Fueron analizadas sesenta muestras de ensilaje pre-fermentado y 60 post-fermentado. El aislamiento de hongos se realizó a través del método de diseminación en superficie en los medios de cultivo diclorán rosa de bengala cloranfenicol, diclorán glicerol 18 % y agar Nash-Snyder. Los aislados de los géneros *Aspergillus, Penicillium* y *Fusarium* fueron identificados a nivel de especie. La determinación de AFB_1 fue realizada por cromatografía de alta eficiencia. Alrededor del 30 % de muestras pre-fermentadas de ensilaje y 55 % pos-fermentadas mostraron recuentos fúngicos por encima de los límites permitidos (1×10^4 UFC g^{-1}). Las especies fúngicas más frecuentes en ambos tipos de muestras fueron *A. flavus, P. citrinum, P. islandicum* y *F. verticillioides*. En promedio, el 32 % de las muestras pre y post-fermentadas fueron positivas para la incidencia de aflatoxina B_1. La presencia de hongos y aflatoxina B_1 en los alimentos destinados a animales demuestra la contaminación. Esta toxina puede afectar la productividad y la salud del animal por lo que este hecho requiere un monitoreo periódico para prevenir la incidencia de hongos toxicogénicos y micotoxinas en la producción animal.

Palabras clave: aflatoxina B_1, cría intensiva de bovinos, ensilaje de sorgo, hongos.

Introduction

Worldwide, the sorghum (*Sorghum bicolor*) is considered the fifth most important crop among the cereals. Silage is a fodder preservation technique achieved through spontaneous lactic acid fermentation under anaerobic conditions. The soluble carbohydrates of the forage are fermented by epiphytic lactic acid bacteria (LAB) producing lactic acid and smaller amounts of acetic acid. In such environment, pH descends to a level in which growth of spoilage microorganisms (including most fungi) is inhibited (Gonzalez Pereyra *et al.,* 2007). Poor management during silage processing or storage can result in oxygen ingress into the silo, causing excessive moisture or dryness, condensation, heating, leakage of rainwater and insect infestation of the silo, leading to undesirable growth of microaerobic acid-tolerant fungi, which may lead to mycotoxins production in this substrate (Dos Santos *et al*., 2003).

Aflatoxins (AFs) are a group of naturally occurring mycotoxins produced by *Aspergillus* fungi, especially *A. flavus* and *A. parasiticus*. Aflatoxin B_1 (AFB_1) has a high carcinogenic potential, especially in liver tissue, and possess an acute toxicity at high concentrations (Khanafari *et al*., 2007). Food contamination with AFs is a global problem, especially in developing countries due to their detrimental impact on human and animal health, which include carcinogenic, mutagenic, teratogenic and immunosuppressive effects (Murthy *et al*., 2005;). In addition to mycotoxins adverse effects on animals, there is also public health concern over the potential transfer of mycotoxins residues to animal-derived food products, such as meat or milk (Hollinger *et al*., 1999).

In Brazil, sorghum showed significant improvement from the 70's, with major expansion in the southern, midwest and southeast mainly, which represent about 90 % of grain sorghum cultivated in Brazil (EMBRAPA, 2009). The cultivated area for silage production, about 160 thousand hectares, is mainly concentrated in the southeast and south (Awika and Rooney, 2004). While grain sorghum is mainly destined to poultry and swine production, sorghum silage and forage grazing are increasingly used for beef and dairy herds (Melo, 2004). In addition, the meat agribusiness has interested in rise the consumption of sorghum in diets for monogastric animals in order to develop their production. The prevalent environmental conditions in Brazil, together with inadequate feed storage provide suitable conditions for fungal development. Although several studies on silage mycotoxins have published worldwide, there is no available data on exposure levels of *Aspergillus* mycotoxins from silage in our country.

Since of the scarcity of information, the aims of the present study were: i) to determine the toxicogenic fungi present in pre and post-fermented sorghum silage, ii) to evaluate the occurrence of AFB_1 in this substrate.

Material and Methods

Sampling

A total of 24 silos of forage sorghum (*S. bicolor*) (12 pre-fermented silo and 12 post-fermented silo) were sampled between June and October 2007 and a further set between February and May 2008. Those silos were located on bovine intensive-rearing (feed-lot) farms in São Paulo State, Brazil. Silage samples (n = 60) from pre and 60 samples from post-fermented silos used to fed beef cattle in Brazil were collected. To ensure a correct sampling, each silo had a linear imaginary division in its length into three equal parts from which primary samples (3 kg) from the upper layer (UL), lower layer (IL), two laterals layer (LL) and central layer (CL) (equidistant at 1.5 m each other) were collected during feed out. Therefore, five points per silo were sampled, obtaining 1 kg of composed samples which were homogenized and quartered to obtain a single laboratory sample. Pre-fermented samples were collected immediately after compaction before the silo closure. Sampling was suspended during the anaerobiosis formation period and reinitiated after compaction period (post-fermentation) 90 days later.

Silos were constructed on the bare ground or over a concrete platform and enclosed with a polystyrene cover. Removal of material for animal feeding was made either by shovelling or using a cutting machine. All of the silages were produced similarly and microbial inoculants were not added. Samples were properly packed in bags and immediately sent to the laboratory. Samples were immediately processed for physical and mycological analyses and kept at -4 °C until mycotoxins analysis.

Physical properties of samples

The pH and dry matter percentage for 100 g of each sample were determined according to Ohyama *et al.* (1975).

Mycological analysis

The quantitative enumeration of fungi as colony-forming units per gram of food (CFU g^{-1}) was performed using the surface-spread method described by Pitt and Hocking (1997). 10 g of each sample were homogenized in 90 mL distilled water solution for 30 min in an orbital shaker. Serial dilutions (10^{-2} to 10^{-5}) were made and 0.1 mL aliquots were inoculated in duplicates onto the media dichloran rose bengal chloranphenicol agar (DRBC) for estimating total culturable fungi (Abarca *et al*., 1994) and dichloran 18 % glycerol agar (DG18) that favors xerophilic fungi development. The plates were incubated at 25 ºC for 5-7 days. All samples were also inoculated onto Nash and Snyder

agar (NSA) to enumerate *Fusarium* species (Nelson *et al*., 1983). Nash-Snyder plates were incubated at 24 °C for 7 days under a 12 h cold white/12 h black fluorescent light photoperiod. Only plates containing 10-100 colonies were used for counting, with results expressed as colony forming units (CFU) per gram of sample. On the last day of incubation, individual CFU g^{-1} counts for each colony type considered to be different were recorded. Colonies representative of *Aspergillus* and *Penicillium* were transferred for sub-culturing to tubes containing malt extract agar (MEA) whereas *Fusarium* spp were transferred for sub-culturing to plates containing carnation leaf agar (CLA). Fungal species were identified according to several keys (Klich, 2002; Nelson *et al*., 1983; and Samson *et al.*, 2000). The results were expressed as isolation frequency (% of samples in which each genera was present) and relative density (% of isolation of each species among the same genera).

Aflatoxin B_1 determination

The extraction of AFB_1 was evaluated according to methodology described by Soares and Rodrigues-Amaya (Soares and Rodrigues-Amaya, 1989). Quantitative evaluation was made using high performance liquid chromatography (HPLC). The detection limit of the techniques for AFB_1 was 1.0 μg kg^{-1}.

Statistical analyses

Statistical analysis of data was by the general linear models model (MLGM). Fungal counts were transformed to $\log^{10}$ (x + 1). Means were compared using Duncan test. Means obtained from CFU g^{-1} mycotoxin analyses were compared using Fisher's protected LSD test. The analysis was conducted using PROC GLM in SAS (SAS, 1997).

Results and Discussion

Chemical and physical properties of samples

Table 1 shows the physical properties of the sorghum samples. The pH mean levels ranged from 6.0 to 6.5 in pre fermented sorghum. While the values of pH, from post fermented silage were from 4.0 to 4.5. In both types of samples, dry matter values were from 38 to 42 %.

Table 1.
Physical properties from pre and post-fermented sorghum in several layer of silo.

Silage section	pH		Dry matter (%)	
	Mean ± SD		Mean ± SD	
	A	B	A	B
UL	6.5 ± 0.8	4.5 ± 0.9	42 ± 0.1	38 ± 0.12
CL	6.0 ± 1.1	4.0 ± 1.0	40 ± 0.1	40 ± 0.10
IL	6.0 ± 0.7	4.0 ± 1.6	38 ± 0.1	38 ± 0.07
LL	6.0 ± 0.3	4.0 ± 1.7	39 ± 0.1	42 ± 0.12

SD: standard deviation; A: pre fermented silage B: post fermented silage UL: upper layer, CL: central layer, IL: lower layer, LL: lateral layer.

Mycological survey

Table 2 shows the fungal counts from pre and post-fermented sorghum in different culture media. Total fungal count analyses from pre-fermented showed that values ranging from 1 x 10^2 (LL) to 4 x 10^4 (CL) CFU g^{-1} and 1 x 10^2 (UL) to 4.4 x 10^4 (UL) CFU g^{-1} in DRBC and DG18, respectively. The lowest count was observed in the lower layer (1.2 x 10^2 CFU g^{-1}), while the higher count was observed in the upper layer (1.4 x 10^6 CFU g^{-1}). 29 and 55 % of pre and post-fermented samples, respectively, were above recommended levels. It was noted that 20 and 12 % of pre and post-fermented sorghum samples, respectively, had counts below 1 x 10^2 CFU g^{-1} in NSA (data not shown).

Table 2.
Fungal counts (CFU g^{-1}) from pre and post-fermented sorghum samples in DRBC and DG18 culture media.

Samples	Levels of silo	Fungal counts (CFU g^{-1}) Mean ± SD and Range		LSD test	Contaminated samples (%) over GMP (2008) limits
		Culture media			
		DRBC	DG18		
Pre fermented	UL	3.2 x 10^3 ± 1.8 x $10^{3\ a}$ (3.1 x 10^2 to 6.1 x 10^3)	1.5 x 10^3 ± 1.9 x 10^3 (1.0 x 10^2 to 4.4 x 10^4)	a	29
	IL	2.9 x 10^3 ± 6.7 x $10^{3\ ab}$ (2.2 x 10^2 to 3.3 x 10^4)	7.2 x 10^2 ± 1.0 x 10^3 (1.2 x 10^2 to 4.3 x 10^4)		
	LL	1.4 x 10^3 ± 1.3 x $10^{3\ ab}$ (1.0 x 10^2 to 3.1 x 10^4)	2.7 x 10^3 ± 8.6 x 10^4 (1.0 x 10^2 to 4.1 x 10^5)		
	CL	3.6 x 10^3 ± 7.2 x $10^{3\ ab}$ (1.3 x 10^2 to 4.0 x 10^4)	1.3 x 10^3 ± 1.2 x 10^3 (1.2 x 10^2 to 4.0 x 10^4)		
Post fermented	UL	8.3 x 10^4 ± 3.8 x $10^{4\ b}$ (1.2 x 10^3 to 1.4 x 10^6)	2.4 x 10^4 ± 3.7 x 10^4 (1.0 x 10^3 to 3.1 x 10^4)	b	55
	IL	2.2 x 10^4 ± 4.1 x $10^{4\ bc}$ (1.2 x 10^2 to 1.3 x 10^5)	3.4 x 10^4 ± 6.5 x 10^5 (1.2 x 10^3 to 6.1 x 10^5)		
	LL	1.3 x 10^4 ± 1.4 x $10^{4\ bc}$ (1.0 x 10^3 to 1.0 x 10^5)	1.4 x 10^4 ± 2.5 x 10^4 (7.2 x 10^3 to 7.1 x 10^4)		
	CL	1.3 x 10^4 ± 3.5 x $10^{4\ bc}$ (2.0 x 10^2 to 4.3 x 10^5)	1.8 x 10^4 ± 3.1 x 10^4 (1.0 x 10^3 to 1.1 x 10^5)		

Mean values of counts ± standard deviation (SD). Minor and major values count. Detection limit: 1x 10^2 CFU g^{-1}. Maximum recommended level: 1 x 10^4 CFU g^{-1} (GMP, 2008). DRBC: dichloran rose bengal chloranphenicol. DG18: dichloran glycerol 18 %. Letters in common are not significantly different according to Fisher's protected LSD test ($p<0.0001$). Fungal counts (CFU g^{-1}) obtained from each culture media at each kind of samples were statistically analyzed separately. UL: upper layer, CL: central layer, IL: lower layer, LL: lateral layer.

Figure 1 shows the isolation frequency (%) of different fungal genera from pre and post-fermented sorghum samples. Yeasts and seven different genera of filamentous fungi were isolated. *Aspergillus* spp was the most frequent in both types of silage, followed by *Cladosporium* spp and *Penicillium* spp in pre-fermented and post-fermented samples. Figure 2 shows relative density of each *Aspergillus*, *Penicillium* and *Fusarium* species isolated from pre and post-fermented samples. *Fusarium verticillioides* (100 %) and was the only specie isolated of *Fusarium* genera in both types of silage. *Aspergillus flavus* was most predominant in pre (80 %) and post (70 %) fermented samples. *Penicillium citrinum* (75 %) was most predominant specie isolated of *Penicillium* genera in both types of silage.

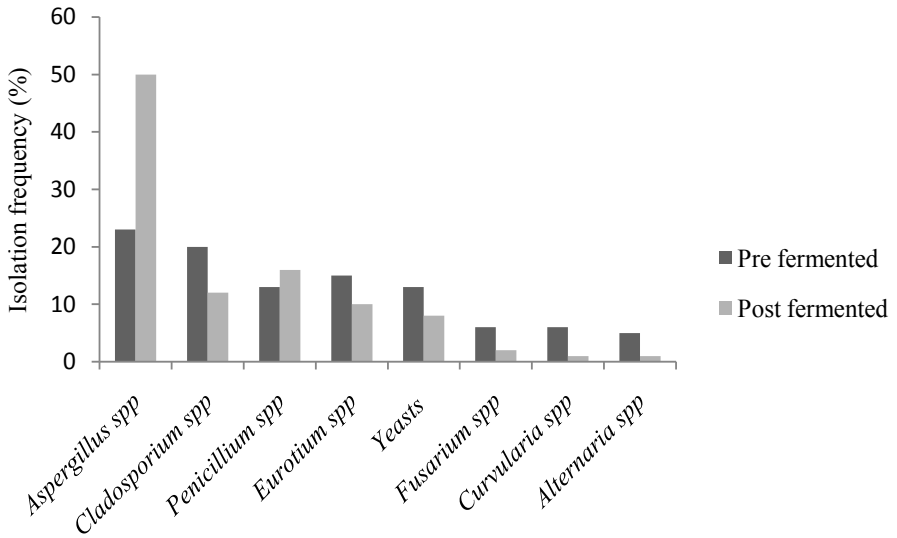

Figure 1. Isolation frequency of fungal genera (%) from pre and post-fermented sorghum samples.

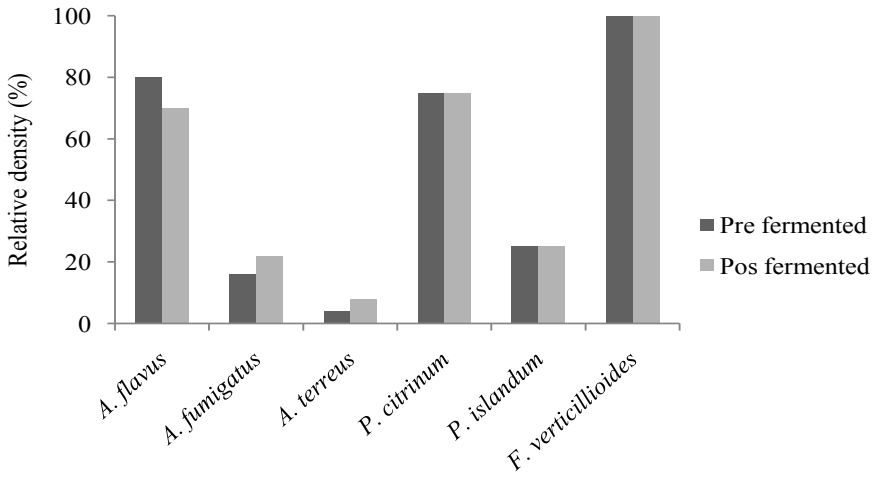

Figure 2. Relative density (%) of *Aspergillus* spp, *Penicillium* spp, and *Fusarium* spp isolated from pre and post-fermented sorghum samples.

Table 3.
Incidence of aflatoxin B_1 in pre and post-fermented sorghum samples.

Samples	Levels of silo	AFB_1 (μg kg^{-1})		
		Mean levels	Range	(%)*
Pre-fermented	UL	2.30[a]	1.04 – 3.44	27
	IL	3.30[a]	1.03 – 5.10	39
	LL	2.33[a]	1.35 – 4.23	31
	CL	3.20[a]	1.10 – 4.34	29
Post-fermented	UL	15.7[b]	2.01 – 25.18	37
	IL	17.6[b]	2.05 – 30.05	32
	LL	15.6[b]	2.35 – 29.78	30
	CL	15.4[b]	2.54 – 24.25	29

*Frequency of contamination (%). Values with letters in common are not statistically significant, according to test of LSD ($p \leq 0.05$). UL: upper layer, CL: central layer, IL: lower layer, LL: lateral layer.

Determination of aflatoxin B_1

Table 3 shows the AFB_1 levels found in pre and post-fermented sorghum samples. Analyzing AFB_1 data, lower layer (IL) samples from pre (39 %) and post (32 %) fermented sorghum samples were most contaminated with values ranging from 1.03 to 5.10 μg kg^{-1}, and 2.05 to 30.05 μg kg^{-1}, respectively. There were significant differences between AFB_1 levels found in samples from pre and post-fermented ($p<0.001$).

The present study shows that fungi and mycotoxin such as AFB_1 are present in pre and post-fermented sorghum used as feed intended for beef cattle in Brazil.

Chemical and physical properties of sorghum silage showed that there was not difference in dry matter comparing pre and post-fermented sorghum. Values of dry matter in this work agree with Neumann et al. (2004) who analyzed sorghum silage for beef cattle in the region of Rio Grande do Sul (Brazil), while differing from other researchers who obtained lower values (Evangelista *et al*., 2005; Rodriguez *et al.*, 1999). The dry matter content is one of the main factors for well preserved silage because it is responsible to affect the type of fermentation and storage. The ideal values of this parameter are between 26 and 38 % with pH around 4.0 (Silva, 2001). The pH difference between pre and post-fermented samples is due to the acidification of carbohydrates present in the raw material by microorganism of this ecosystem. In this work, the pH values in post-fermented sorghum were from 4.0 to 4.5 after 90 days of fermentation. Other researchers working with the same type of substrate found a pH 3.41 and 3.65 when analyzing silage with 56 days post-fermentation (Evangelista *et al*., 2005; Rodriguez *et al*., 1999). Generally, fungal growth and mycotoxin production oc-

curs when the herbage is inadequately sealed from air, usually when it is not well packed; the oxygen tension is high and pH values range from 6.0 to 7.0 (Gotlieb, 1997).

In this study, a high mycological contamination was found. All post-fermented layers had counts over the proposed limits (1×10^4 CFU g^{-1}) (GMP, 2008). These results suggest a high fungal activity that could affect the palatability of feed and reduce the animal nutrients absorption, determining a low quality substrate (Ogundero, 1989; Martins and Martins, 2001). *Aspergillus* was the prevalent genera in pre and post-fermented sorghum. This result is consistent with other studies in São Paulo State (Silva *et al*., 2000; Lasca *et al.*, 1986) and other researchers who have studied the fungal contamination in sorghum from Botswana and Argentina (Nkwe *et al.*, 2005; González *et al*., 1997). *Aspergillus flavus* showed the highest relative density among *Aspergillus* species, followed by *A. fumigatus*. These results agree with Gonzalez Pereyra *et al.* (2007) and El-Shanawany *et al.* (2005) who studied corn silage in Argentina and Egypt, respectively. Although a low density of *A. fumigatus* strains was found, their presence means a dual hazard from the ingestion of pathogenic spores and from potential mycotoxin production such as gliotoxin, fumigaclavine A, fumigaclavine C and several fumitremorgens. Within *Penicillium* and *Fusarium* genera, the most frequent isolated species in this study were *P. citrinum* and *F. verticillioides*. Nkwe *et al.* (2005) isolated a low incidence of *Penicillium* spp and *P. citrinum* was the predominant. Silva *et al*. (2000), unlike our results, in addition to *F. verticillioides* (25 %) isolated *F. subglutinans* (7.1 %), *F. semitectum* (5.7 %) and *F. proliferatum* (2.1 %), while other researchers obtained similar results to ours (Nkwe *et al*., 2005; González *et al*., 1997).

Animal feed is frequently contaminated simultaneously by several fungi, which are able to produce different kinds of toxins each. In animal production, this situation brings not only bad health to animals but also low production. Pre and post-fermented samples shown AFB_1 levels, which increased significantly in the post-fermented sorghum silage samples. AFB_1 levels present in most of post-fermented samples were higher than 20 ng g^{-1}, more than the recommended limit (GMP, 2008). Silva *et al*. (2000) found that 12.8 % of the sorghum grain samples from São Paulo (Brazil) were contaminated with AFB_1 at levels ranging from 7 to 33 µg kg^{-1}. Similar results were obtained in Ethiopia by Ayalew *et al.* (2006), where they studied 86 samples of sorghum grain and 6.1 % were positive for this toxin at average concentration of 5.9 µg kg^{-1}. Others researchers did not detect AFB_1 levels, despite of the isolation of *A. flavus* studied in 46 sorghum malt samples (Nkwe *et al*., 2005). In this study, *A. flavus* frequency and AFs contamination showed a negative correlation. Regarding to low dietary level of mycotoxins, Hamilton (1984) also reported the fact that any level of mycotoxins carries risk of economic losses and that is impossible to define a safe level under field conditions. Oude Elferink *et al*. (2000) reported that conditions under mycotoxin production in silage remain uncertain. The biological effects of mycotoxins depend on the ingested amount, number of occurring mycotoxins, and time of exposure and animal sensitivity. Moreover, the mycotoxin effects are not only amplified by stress production but also high in intensively reared cattle destined to meat or milk production (Binder, 2007; Yiannikouris and Jouany, 2002).

Worldwide, reports on mycobiota and mycotoxin contamination of sorghum silage are scarce. Also, this is the first work that evaluates fungi and mycotoxins in pre and post-fermented sorghum silage used for beef cattle consumption. The presence of mycotoxins in these substrates indicates the existence of contamination. These results reveal the need for periodic monitoring of sorghum silage to avoid animal production impairment and hazards to animal and human health.

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