STUDY ON THE MYCOBIOTA AND MYCOTOXINS OF COMMERCIAL EQUINE FEEDS IN RIO DE JANEIRO, BRAZIL*

ESTUDO SOBRE A MICOBIOTA E AS MICOTOXINAS EM RAÇÕES COMERCIAIS DESTINADAS À ALIMENTAÇÃO DE EQÜINOS NO RIO DE JANEIRO, BRASIL

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Colonization of feedstuffs and agricultural commodities by mycotoxins producer moulds is a significant problem worldwide. Contamination of feed ingredients, increases the risk of contamination of the finished products such as manufactured commercial feeds. Studies evaluating the risk of mycotoxins in the equine environment are scarce. The aims of this study were to evaluate the mycobiota present in commercial equine feeds and to determine total aflatoxins(AFs) and fumonisins (FBs). Thirty samples of 21 commercial equine feeds were collected at random from 5 different equestrian centers located in Rio de Janeiro, Brazil. Total fungal counts (CFU g^{-1}) of equine feeds ranged from not detectable (ND) to 1.3 x 10^6 CFU/g onto DRBC medium. *Penicillium* spp. (52%) was the prevalent genera, followed by *Aspergillus* spp. (32.5%). *Aspergillus niger* aggregate (60%) and *Aspergillus flavus* (22%) showed the highest relative densities. Thirteen *Penicillium* spp. were isolated. All samples (100%) were contaminated with AFs at levels that varied between 1 and 44 μg/Kg. The 65% of these samples were positive to FBs contamination at levels ranging from 0.2 to 8.5 μg/g. Monitoring mycological and mycotoxicological quality of feed ingredients and commercial feedstuffs used in equine husbandry are critical for improving animal production and performance.

KEY WORDS. Equine, feed, mycobiota, mycotoxins.

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RESUMO. A colonização de matérias-primas e produtos agrícolas por fungos produtores de micotoxinas é um problema significativo em todo o mundo. A contaminação dos ingredientes das rações aumenta o risco do produto acabado como as rações comerciais manufaturadas. Estudos avaliando o risco de micotoxinas no ambiente eqüino são escassos. Os objetivos deste estudo foram avaliar a micobiota presente em rações comerciais para eqüinos e determinar aflatoxinas (AFs) e fumonisinas (FBs) totais. Trinta amostras de 21 marcas comerciais foram coletadas, ao azar, de 5 diferentes centros hípicos localizados no Rio de Janeiro, Brasil. As contagens totais de fungos (UFC g^{-1}) das rações de equinos apresentaram intervalos entre não detectado (ND) a 1,3 x 10^6 UFC/g no meio DRBC. *Penicillium* spp. (52%) foi o gênero prevalente, seguido por *Aspergillus* spp. (32,5%). *Aspergillus niger* agregados (60%) e *Aspergillus flavus* (22%) apresentaram as maiores densidades relativas. Treze espécies de *Penicillium* foram isoladas. Todas as amostras (100%) estavam contaminadas com AFs com níveis que variaram entre 1 e 44 μg/Kg. Um total de 65% das amostras estavam positivas para a contaminação por FBs com níveis que variaram entre 0,2 a 8,5 μg/g. O monitoramento micológico e micotoxicológico da qualidade dos ingredientes e rações comerciais usados na alimentação eqüina são críticos para incrementar a produção e performance animal.

PALAVRAS-CHAVE. Eqüinos, rações, micobiota, micotoxinas.

INTRODUCTION

Colonization of feedstuffs and other agricultural commodities by mycotoxins producer moulds is a significant problem worldwide. Mycotoxins are mould secondary metabolites that have detrimental effects on humans, animals and crops, resulting in illness and economic losses (Hussein & Brasel, 2001). Mould and mycotoxin contamination of crops and grainsused as feed ingredients, increases the risk of contamination of the finished products such as manufactured commercial feeds.

Aflatoxins (AFs) - produced by *Aspergillus flavus* and *A. parasiticus* - frequently contaminate a wide variety of inadequately stored food commodities. They are carcinogenic and they are potent liver toxins (Cast, 2003). Aflatoxin B_1 - the most prevalent - has been classified as 1B human carcinogen by the International Agency for Research on Cancer (Iarc, 2002). In horses, AFs exposure has lead to many clinical symptoms including body weight loss, liver damage and centrilobular hepatic disease among others (Angsubhakorn et al., 1981; Bortell et al., 1983; CAST, 2003). Several outbreaks of acute equine aflatoxicosis have been reported (Angsubhakorn, 1981; Aller et al., 1981; Bortell et al., 1983; Vesonder et al., 1991).

Fumonisins (FBs) are a family of mycotoxins produced primarily by *Fusarium verticillioides* and *F. proliferatum* that are prevalent contaminants in corn (EHC, 2000). Fumonisin B_1 can cause leucoencephalomalacia in horses (Marasas et al., 1988), porcine pulmonary edema (PPE) in pigs (Harrison et al., 1990), hepatoxicity and kidney cancer in rats (Gelderblom et al., 1988; IARC, 2002). Equine leucoencephalomalacia (ELEM) is a neurotoxic disease typified by staggers, stupor, lameness and seizure (due to brain necrosis) and death (Newman & Raymond, 2005).

The nature of the horse farm makes the equine different from other livestock species. They are not bred for growth or meat yield and they have longer lifespan. In most cases horses are bred for athletic performance, conformation, temperament, beauty and/or durability (Newman & Raymond, 2005). Studies evaluating the risk of mycotoxins in the equine environment are scarce. Recently, we have reported the first research on fungi and significant mycotoxins in equine feed in Brazil and Argentina (Keller et al., 2007; González Pereyra et al., 2007). The equine population in Brazil has increased in the latter years, being over 5.8 millions by 2003 and most of them were located in the Southeast region. One of the main activities involving horses is athletic performance. There is a great interest in conducting studies so that performace of these animals can be improved.

The aims of this study were 1) to evaluate the mycobiota present in commercial equine feeds and 2) to determine total AFs and FBs, the main mycotoxins that affect equine health in the Rio de Janeiro, Brazil.

MATERIALS AND METHODS

Feed samples

Thirty samples of 21 commercial equine feeds were collected at random from April 2006 to March 2007, from 5 different equestrian centers located in Rio de Janeiro, Brazil. The samples were grouped according to manufacturer in: light effort (LE), moderate efforts (ME) and intense effort (IE), based

in the composition according to the kind of animal they were intended.

The primary samples (4 kg each) were homogenized and quartered to get 1 kg laboratory samples. An aliquot of 10 g was separated for the analysis of the mycobiota and the rest was milled and stored at 4ºC for mycotoxins analyses.

Mycological survey

Total mould counts of equine feed samples were performed onto dichloran rose bengal chloranphenicol agar (DRBC) (water activity (a_W) 0.99), that is a general media used for estimating total culturable mycobiota (Abarca et al., 2001), dichloran 18% glycerol agar (DG18) (a_W 0.97), which has low a_W and favours xerophilic fungi development (Pitt & Hocking, 1997) and Nash Snyder agar (NSA), that is a selective medium for *Fusarium* species. Quantitative enumeration of fungal propagules in solid media was done using the surface-spread method. Ten grams of each sample were homogenized in 90 ml 0.1% peptone water solution for 30 min in an orbital shaker. Serial dilutions (10^{-2} to 10^{-4}) were made and 0.1 ml aliquots were inoculated in duplicates onto the media described above. The plates were incubated at 25ºC for 7-10 days. Only plates containing 10-100 colony-forming units (CFU) were used for counting, with results expressed as CFU per gram of sample. On the last day of incubation, individual CFU/g counts for each colony type considered to be different were recorded. Colonies presumable to be *Fusarium* spp. were transferred to tubes containing V8 juice agar (V-8J) and Petri dishes containing carnation leaf agar (CLA) and other colonies were transferred to tubes containing malt extract agar (MEA). Fungal genera and species were identified according to 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 strains of the same genera).

Mycotoxin analyses

Total aflatoxins and fumonisins were quantified in equine feed samples using commercial enzyme-linked immunoassay (ELISA) kits (Beacon Analytical Systems Inc.). The procedure was done following the manufacturer's directions. Samples were extracted with a mixture of methanol and water into a blender and then filtered. The filtrate was tested for total aflatoxins and fumonisins content using the immunoassay method.

RESULTS

Mycological survey

Total fungal counts (CFU g^{-1}) of equine feeds ranged from not detectable (ND) to 1.3 x 10^6 CFU/g in LE, from ND to 6 x 10^3 CFU/g in ME and from ND to 1.0 x 10^3 CFU/g in IE feeds onto DRBC medium. Total fungal counts performed onto DRBC, DG18 and *Fusarium* spp. counts performed onto NSA medium are shown in Table 1. Results were similar when compared counts from both culture media (P<0.05). The highest counts were found in LE feed samples. *Penicillium* and *Aspergillus* were the prevalent genera and were found at high counts especially in DG18 medium.

Penicillium spp. (52%) was the most frequently isolated genera, followed by *Aspergillus* spp. (32.5%). Other genera – *Moniliella* spp., *Eurotium* spp., *Cladosporium* spp., *Fusarium* spp., *Rhizopus* spp., *Mucor* spp. – were isolated with lower frequencies (Figure 1).

A total of six *Aspergillus* species were isolated from the equine feeds. *Aspergillus niger* aggregate (60%) showed the highest relative density, followed by *Aspergillus flavus* (22%) (Figure 2). Thirteen *Penicillium* spp. were isolated from these samples, being *P. corylophilum* the prevalent species (20 %),

Table 1. Total fungal counts ranges and mean values found in three different categories of commercial equine feeds.

Feed category	Total fungal counts ranges (CFU/g)		
	DRBC	DG18	NSA
Light effort (n=15)	ND - 1.7 x 10^6 (Mean = 1.3 x 10^5)	ND - 3.8 x 10^6 (Mean = 2.7 x 10^5)	ND - 0.9 x 10^4 (Mean = 0.9 x 10^3)
Moderate effort (n=08)	ND - 0.6 x 10^4 (Mean = 1.5 x 10^3)	ND - 2.2 x 10^4 (Mean = 0.5 x 10^4)	ND - 0.2 x 10^4 (Mean = 0.4 x 10^3)
Intense effort (n=07)	ND - 1.0 x 10^3 (Mean = 0.3 x 10^3)	ND - 1.0 x 10^3 (Mean = 0.3 x 10^3)	ND - 1.0 x 10^2 (Mean = 0.1 x 10^2)

ND: not detectable. Limit of detection: 10^2 CFU g^{-1}.

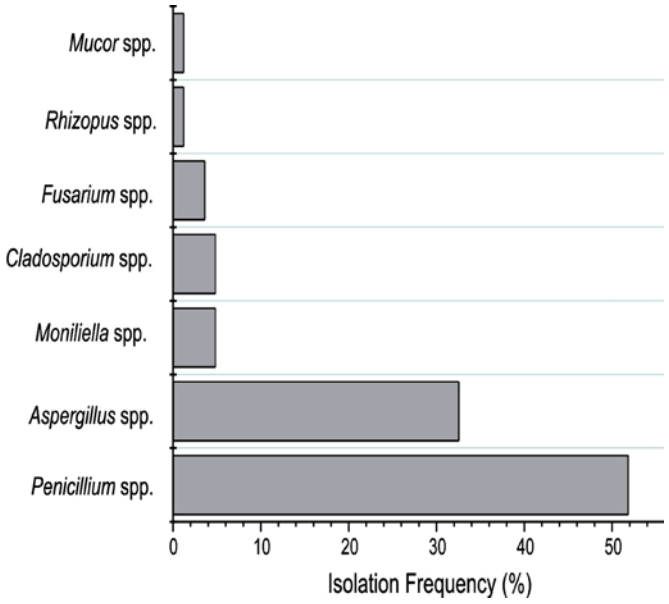

Figure 1. Isolation frequency (%) of the different fungal genera found in commercial equine feed samples collected in Rio de Janeiro, Brazil.

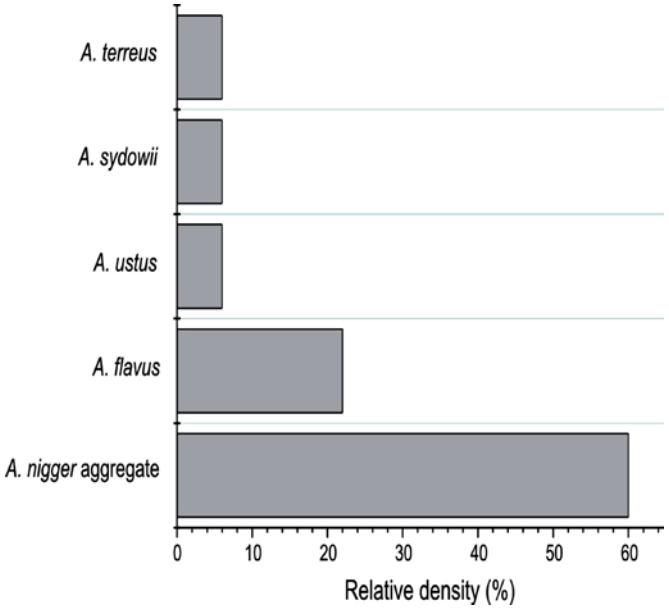

Figure 2. Relative density (%) of the different *Aspergillus* spp. found in commercial equine feed samples collected in Rio de Janeiro, Brazil.

followed by *P. fellutanum* and *P. funiculosum* (14% each) (Figure 3).

Mycotoxins analyses

Mycotoxins levels detected in the different analyzed equine feeds are shown in Table 2. All of the samples (100%) were contaminated with AFs at levels that varied between 1 and 44 µg/Kg (ppb). The 65% of these samples were positive to FBs contamination at levels that varied from 0.2 to 8.5µg/g (ppm).

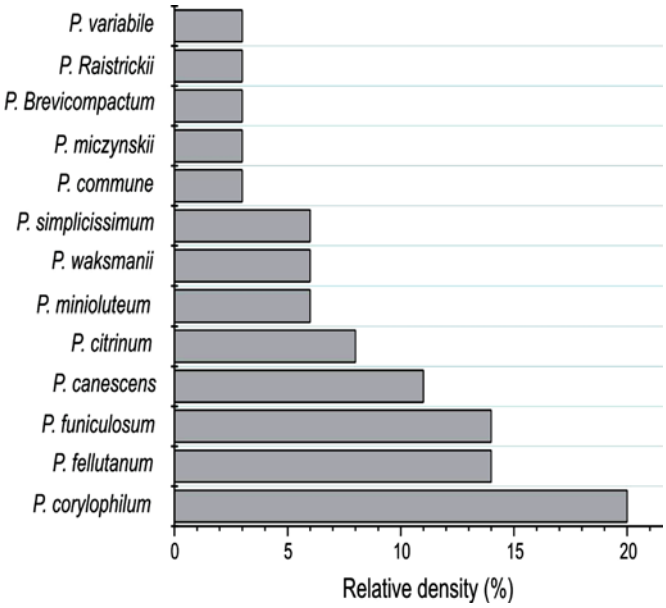

Figure 3. Relative density (%) of the different *Penicillium* spp. found in commercial equine feed samples collected in Rio de Janeiro, Brazil.

Table 2. Total aflatoxins (AFs) and fumonisins (FBs) level ranges detected in commercial equine feed samples.

Mycotoxins	Level range	Contaminated samples (%)
AFs	1 – 44 µg/Kg	100
FBs	ND – 8.5 µg/g	63

ND: not detected.

DISCUSSION

The mycobiota present in commercial equine feed and the main mycotoxins that affect equine health were studied during this research. Total fungal counts found in some of the analyzed commercial feeds exceeded the hygienic quality limit proposed for animal feeds and feed ingredients (1 x 10^4 CFU/g) (Gmp, 2008). These results are comparable with those obtained in previous studies performed in Brazil and Argentina where total fungal counts ranged from < 1 x 10^2 to > 1 x 10^5 in equine feeds and raw materials used as feedstuffs (González Pereyra, et al., 2007; Keller et al., 2007). Most samples of commercial feeds analyzed in this study showed lower fungal counts probably due to pelletization process. It is known that high temperatures, humidity and pressure characteristics of the pelletization process destroy most of the conidia present in feed. The presence of high counts in finished palletized feed suggests the utilization of poor quality ingredients in the manufacturing of the feed.

Though counts were low, potentially toxigenic species – *A. flavus* and *A. nigger* aggregate – were isolated from these substrates. These species have

already been isolated from equine feedstuffs in previous studies (González Pereyra et al., 2007; Keller et al., 2007). The presence of ochratoxin A was not evaluated in this study and 100% of the analyzed samples were contaminated with AFs suggesting a direct relation between the presence of the producer fungi and the natural occurrence of the toxin in the feed. None of the samples exceeded the maximum tolerated levels for total AFs (50 ppb) (FAO, 2004). Yet, the toxin was present. Aflatoxins and FBs co-occurred in 63% of the samples. A synergistic toxic response may be possible in animals when simultaneous exposure occurs (Leung et al., 2006). Only one sample (3%) showed FBs levels higher than the limits recommended by the UE (5 ppm) (Official J EU, 2006). Even though the levels were low, the presence of the toxin indicated the producer fungi were indeed contaminating the feed and that the product was exposed to environmental conditions for FBs production. During the sampling period, from October 2005 to March 2006, high mean temperatures were registered, between 28º and 40ºC. This excessive heat may have been the factor that restricted *Fusarium* spp. proliferation, resulting in low counts in NSA medium.

The results obtained in the present study indicate the importance of mycological survey for estimating potential toxicological problems in equine feeds. Official limiting values of mycotoxins have not been established for horses in particular as they have been for other animals such as poultry and dairy cows. However, it is known that low levels of exposure over long periods can elicit chronic or sub-chronic toxicological manifestations affecting performance or breeding ability without the appearance of overt signs of disease (Newman & Raymond, 2005). Monitoring mycological and mycotoxicological quality of feed ingredients and commercial feedstuffs used in equine husbandry are critical for improving animal production and performance.

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REFERENCES

Abarca, M.L.; Bragulat, M.R.; Castella, G. & Cabañes, F.J. Mycoflora and aflatoxin-producing strains in animal mixed feeds. *J. Food Prot*., 57:256-258, 1994.

Aller, W.W.Jr.; Edds, G.T. & Asquith, R.T. Effects of aflatoxins in young ponies. *Am. J. Vet. Res.*, 42:2162-2164, 1981.

Angsubhakorn, S.; Poomvises, P. & Newberne, P.M. Aflatoxicosis in horses. *J. Am. Vet. Med. Assoc*., 178:274-278, 1981.

Bortell, R.; Asquith, R.L.; Edds, G.T.; Simpson, C.F. & Aller W.W. Acute experimentally induced aflatoxicosis in the whealing pony. *Am. J. Vet. Res*., 44**:**2110-2114, 1983.

CAST. *Mycotoxins: risks in plants animal and human systems*. Ames, Council for Agricultural Science and Technology. *Task Force Report* Nº139, 2003.

Dalcero, A.; Magnoli, C.; Chiacchiera, S.; Palacios, G. & Reynoso, M. Mycoflora and incidendence of aflatoxin B1, zearalenone and deoxinyvalenol in poultry feeds in Argentina. *Mycopathologia*, 137**:**179-184, 1997.

EHC. *Environmental Health Criteria 219: fumonisin B1, International Programme on Chemical Safety (IPCS; UNEP, ILO and WHO).* Geneve, Eds. W.H.O., 2000.

EU. Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding (2006/576/EC). Official J European Union (2006). Available on : < URL:http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:229:0007:0009:EN:PDF.>. Acess on : Aug. 17, 2006.

FAO, Worldwide Regulations for Mycotoxins in Food and Feeds in 2003. Food and Nutrition Paper 81, Roma, FAO Organization of the United Nations, 2004.

Gelderblom, W.C.A.; Jaskiewic, K. & Marasas, W.F.O., 1988. Fumonisins-novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme. Appl. Environ. Microbiol.*, 54:1806-1811.

González Pereyra M.L.; Tissera J.; Manteña C.; Cavaglieri L.R.; Keller K.M.; Rosa C.A.R., Magnoli C. & Dalcero A.M.. Mycotoxicological Quality of Equine Feed in Argentina. 10th Congress of World Equine Veterinary Association. Moscou, Russia, 2008.

Marasas, W.F.O.; Kellerman, T.S.; Gelderblom, W.C.A.; Coetzer, J.A.W.; Thiel, P.G. & Van Der Lugt, J.J. Leukoencephalomalacia in a horse induced by fumonisin B_1 isolated from *Fusarium moniliforme.* Ondertepoort J. Vet. Res., 55:197-203, 1988.

GMP, Good Manufacturing Practice. Certification Scheme Animal Feed Sector 2006, Including Residue Standards (Version 28 March 2008), 2008.

Hussein, S.H. & Brasel, J.M. Toxicity, metabolism and impact of mycotoxins on humans and animals. *Toxicology*, 167:101-134, 2001.

IARC. International Agency for Research of Cancer 82:171-301. 2002.

Keller, K.M.; Queiroz, B.D.; Keller, L.A.M.; Ribeiro, J.M.M.; Cavaglieri, L.R.; González Pereyra, M.L.; Dalcero, A.M. & Rosa, C.A.R. The mycobiota and toxicity of equine feeds. *Vet. Res. Comm*., 31:1037-1045, 2007.

Klich, M.A. *Identification of common Aspergillus species.* CBS, Utrecht, 2002.

Leung M.C.K.; Díaz-Liano G. & Smith T.K. Mycotoxins in pet food: a review on worldwide prevalence and preventative strategies. *J. Agric. Food Chem*., 54: 9623-9635, 2006.

Nelson, P.E.; Toussoun, T.A. & Marasas W.F.O. *Fusarium* species: An illustrated manual for identification. Pennsylvania State University Press, Philadelphia, 1983.

Newman, K.E. & Raymond S.L., 2005. *Effects of Mycotoxins in Horses*, p.57-76. In. Diaz, D.E. (Ed.), *The Mycotoxin Blue Book*. Nottingham University Press, Nottingham, 2005.

Pitt J.I. & Hocking A.D. Fungi and Food Spoilage 2nd ed., Blackie Academic Press, London, 1997.

Samson, R.A; Hockstra, E.S.; Frisvad, J.C. & Filtenborg, O. *Introduction to food and airborne fungi*. The Netherlands: Centaalbureau Voorschimmelculturs. Ponson & Looyen, Wageningen Press, Utrecht, 2000.

Vesonder, R.; Haliburton, J.; Stubblefield, R.; Gilmore, W. & Peterson, S. *Aspergillus flavus* and aflatoxins B_1, B_2, and M_1 in corn associated with equine death. *Arch. Envioron. Contam. Toxicol*., 20:151-153, 1991.