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Mycotoxicological studies of an *Aspergillus oryzae* strain

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The safety of *Aspergillus oryzae* used in industrial fermentations of food-grade products has long been recognized. However, production of fungal toxic secondary metabolites is strain-specific and environment-dependent. For these reasons, the present study aimed to conduct a mycotoxicological study of the strain H/6.28.1 of *A. oryzae* (from the collection of the Cuban Institute of Sugar Cane Derivatives Research), intended for use as microbial feed additive for ruminants. Analysis of mycotoxins in fungal culture extracts was carried out by thin layer chromatography and high-performance liquid chromatography. Results show that the strain under study does not produce detectable levels of aflatoxins B_1, B_2, G_1, G_2 and "ochratoxin". Cyclopiazonic acid was detected at level of 14.47 $\mu g \cdot ml^{-1}$, which has negligible toxicology significance for animal health. It is concluded that the *A. oryzae* evaluated could be used as a feed additive for ruminants.

Key words: Aflatoxins, ochratoxin A, cyclopiazonic acid, *A. oryzae*, feed additive.

INTRODUCTION

In the last decades, nutritionists have shown great interest in a wide variety of microorganisms that, when added in small quantities to the diet, improve the health and the productivity of animals (Kamra and Agarwal, 2004). This is particularly true for the conidial fungus *Aspergillus oryzae*, which have been shown to produce significant improvements in the performance of ruminants (Wiedmeier et al., 1987; Gómez-Alarcón et al., 1991; Humphry et al., 2002; Kim et al., 2006; Di Francia et al., 2008).

The safety of any microbial product intended for animal feed should be carefully evaluated. The primary issue in the safety evaluation of these microbial feed additives is its toxigenic potential, specifically the possible synthesis of toxins by a particular strain that can cause health disorders in the animals after ingestion through the diet (Pariza and Johnson, 2001). *A. oryzae* is considered as "generally recognized as safe" by the U.S. Food and Drug Administration, and is used in several fermented foods and enzyme preparations (Beuchat, 2001). However, mycotoxin production by some strains has been reported (Blumenthal, 2004). On the other hand, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommends that enzyme preparations derived from fungal sources should be evaluated for those mycotoxins that are known to be produced by strains of the species used in the production of the enzyme preparation or related species (Food and Agriculture Organization, 2001). In this context, the aim of the present study was to determine the ability of an *A. oryzae* strain to produce some mycotoxins that could be produced by the species, before using it as a microbial feed additive in ruminant's diets.

MATERIALS AND METHODS

A strain of *A. oryzae* (H/6.28.1, belonging to the collection of the Cuban Institute of Sugar Cane Derivatives Research) was used

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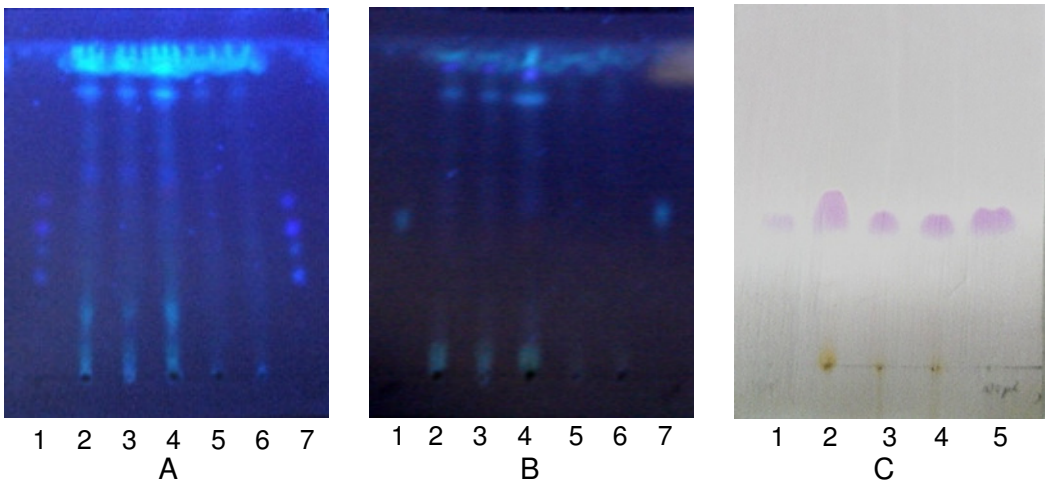

Figure 1. Thin layer chromatographic plates from samples of culture material of *Aspergillus oryzae* showing spots of: (A) aflatoxins B_1, B_2, G_1, and G_2 (spots 1 and 7: standards; spots 2-5: samples); **(B)** ochratoxin A (spots: 1 and 7: standards; spots 2-5, samples **(C)** cyclopiazonic acid (spots 1 and 5: standards; spots 2-4: samples).

The strain was previously isolated from soil and stored in Czapek agar (pH 6.8) at 4°C. Confirmation of the strain identity was accomplished by DNAr digestion in the Spanish Collection of Type Cultures (CECT) from the University of Valencia.

The *A. oryzae* strain was grown for 10 days at 25°C on Czapek yeast autolysate agar plates (Klich and Pitt, 1988) prepared from Czapek-Dox agar added with 5 gl^{-1} yeast extract (Difco). The combined agar and culture were then transferred to erlenmeyers of 250 ml and the mycotoxins were extracted with chloroform/methanol (2:1, v/v; 50 ml per plate) by shaking for 30 min. The mixture was filtered through anhydrous sodium sulphate. The filtrate was evaporated to dryness in a water bath at 40°C under air stream (López-Díaz et al., 2001).

The dried extracts were dissolved in 1 ml chloroform and small aliquots (25 µl) were screened by spotting onto silica gel plates (Merck) along with standard solutions of the following mycotoxins under investigation: aflatoxins (AF) B_1, B_2, G_1, G_2, ochratoxin A (OA) and cyclopiazonic acid (CPA). The standards and the samples were spotted at 1.0 cm from the bottom of the silica gel plates, and spaced 1.0 cm between each one. In the case of aflatoxins, 5 and 10 µl of the standard solution containing 1.0 µg ml^{-1} of each AF were spotted. For OA, 10 and 15 µl of the standard solution (2.0 µg ml^{-1}) were spotted. The mobile phase consisted in a mixture of ether:methanol:water (96:3:1, v/v/v) (Soares and Rodrigues-Amaya, 1989), being the toxins detected by the presence of fluorescent spots of AF`s or OA under ultraviolet (UV) light (366 nm). For CPA, 50 and 100 µl of the standard solution (4.0 µg ml^{-1}) were spotted, and a mobile phase consisted of ethyl acetate:2-propanol:sodium hydroxide (50:15:10, v/v/v) was used (Lansden, 1986). When the chromatographic run for CPA finished, the silica gel plate was dried at 35°C for 3 min and sprayed with Ehrlich's reagent (Lansden, 1986), for observation of CPA purple spots after about 4 min. The spots remained evident for approximately 7 min.

Quantification of the mycotoxins was accomplished in a Shimadzu 10VP liquid chromatographic system equipped with a 10 AXL fluorescence detector (excitation at 360 and 365 nm and emission above 440 and 450 nm, for aflatoxin and OA, respectively. A Phenomenex (Torrance, CA, USA) Sinergy C18 column (4.6 x 150 mm, 4 µm) and a Shimadzu Shim-Pack CLC G-ODS precolumn (4 x 10 mm) were used. A total of 20 µl of sample extracts were injected for determination of aflatoxin. The samples were previously derivatizated with 100 µl of acetic acid and 200 µl of n-hexane and filtered through a membrane (PTFE, 0.45 µm, Millex, Millipore). The mobile phase consisted on a mixture of acetonitrile:water:methanol (60:20:20, v/v/v) with a flow rate of 1.0 ml min^{-1}. Under these conditions, the retention times for aflatoxins were: G1: 4.275 min; B1: 5.136 min; G2: 7.278 min; B2: 9.420 min. Determination of OA determination was developed under the same condition, but the mobile phase consisted on a mixture of methanol-water-acetic acid (60:40:1, v/v/v) with a flow rate of 1.2 ml min^{-1}. A total of 20 µl of sample extracts, previously filtered, were injected. The retention time for OA was approximately 15.219 min. A calibration curve of CPA was prepared using standard CPA solutions at concentrations of 2.41; 4.82 and 9.63 µg·ml^{-1}. A UV visible SPD 10AVP detector, at 279 nm, was used. Chromatography conditions were as follows (Urano et al., 1992): 100% of methanol:water (7:3, v/v) (solvent A) during 5 min, later linear gradient from 100% of solvent A to 100% methanol:water (7:3, v/v) with 4 mM $ZnSO_4x7H_2O$ (solvent B) in 10 min, followed by 100% of solvent B for 5 min. The return of the gradient to 100% of solvent A was reached in 15 min. A total of 20 µl of sample extracts, previously filtered, were injected at a constant flow rate of 1.0 ml min^{-1}. Under these conditions, retention time for CPA was approximately 5.8 min.

RESULTS AND DISCUSSION

The thin layer chromatography revealed that the strain under study did not produce aflatoxins B_1, B_2, G_1 and G_2 and OA (Figures 1, A and B). These results were confirmed with the HPLC (Figures 2 and 3). The detection limit for each aflatoxin (B_1, B_2, G_1, G_2), OA and CPA were 0.5 ng·g^{-1}, 8.7 ng·g^{-1} and 5.0 µg·g^{-1} of culture material, respectively (Table 1).

The non-occurrence of aflatoxins in *A. oryzae* cultures was demonstrated by other authors (Murakami, 1971; Kusumoto et al., 1990). Early studies on the factors determining the aflatoxin production focused on physiological aspects of strains, including growth temperature, age of the culture and nutrient components in the medium (Reddy et al., 1979). The *A. oryzae* strain studied was

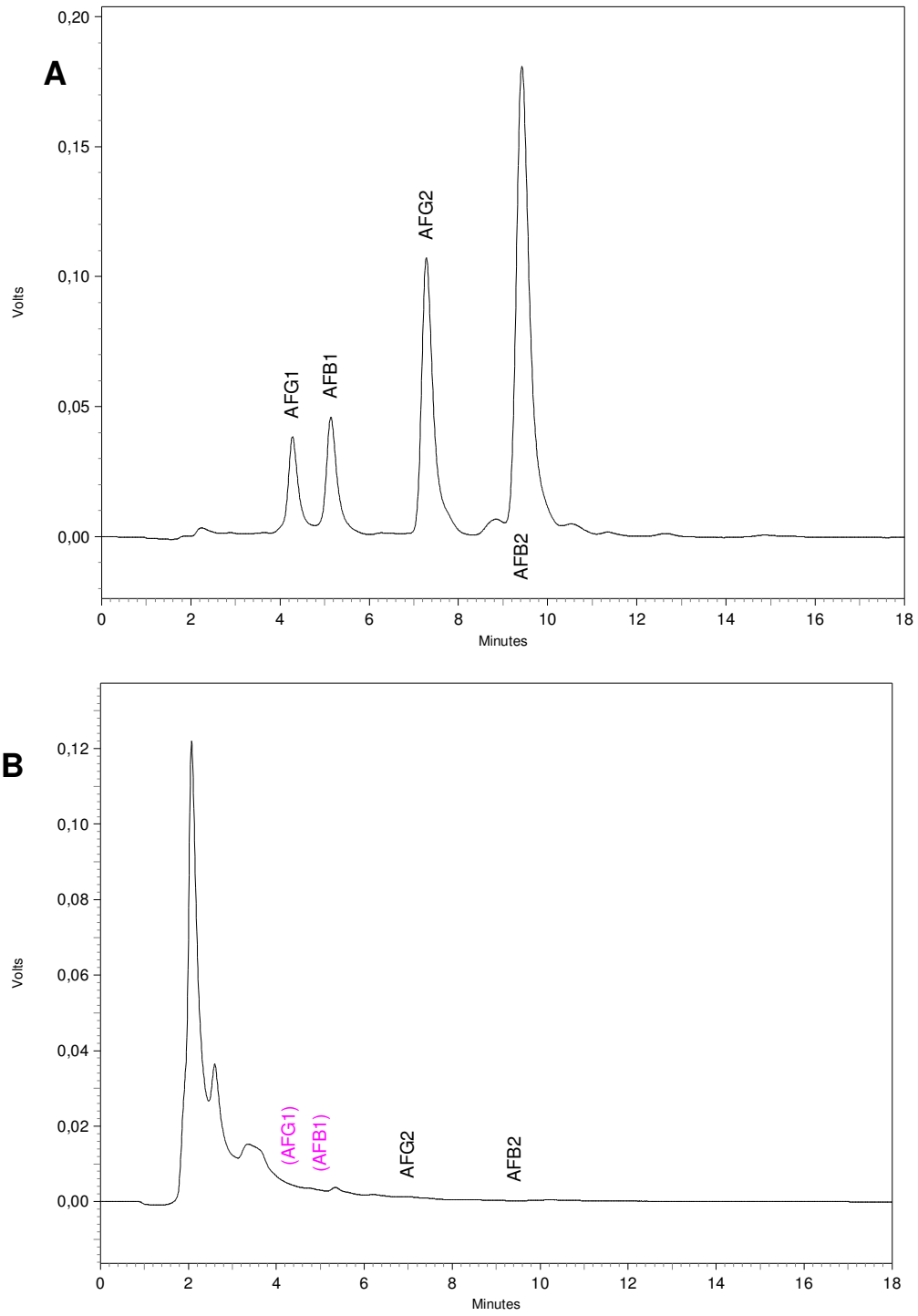

Figure 2. Chromatograms showing (A) Aflatoxins B_1, B_2, G_1, and G_2 standards (20.0 ng·ml^{-1} of each aflatoxin); (B) Sample of culture material of *Aspergillus oryzae*.

cultured under favorable conditions for the synthesis of aflatoxins (López-Díaz et al., 2001). Although in the present study no genetic approach was used, the absence of aflatoxin production by the strain evaluated could be related to its genetic characteristics. Previous work showed a number of mutations within the aflatoxin biosynthesis gene homolog cluster in *A. oryzae* relative to the *A. flavus* sequence, including deletions, frameshift mutations, and base pair substitutions, which induce inactivation at the protein level and consequently impair the aflatoxin synthesis (Kiyota et al., 2011). Aflatoxin production in *A. oryzae* was reported by some authors (Adebajo, 1992; El-Kady

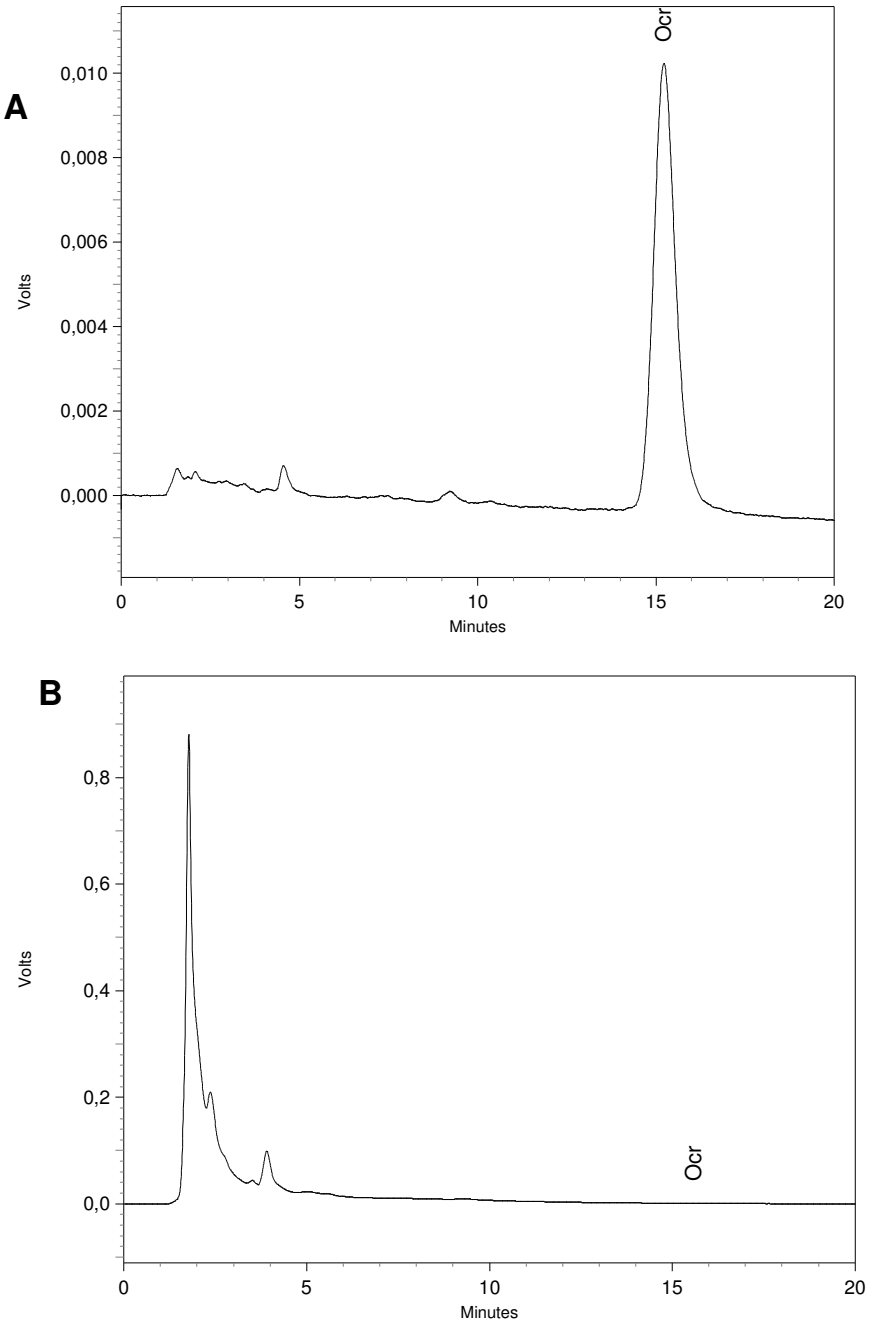

Figure 3. Chromatograms showing **(A)** Ochratoxin A standard (402.8 ng·ml^{-1}); **(B)** sample of culture material of *Aspergillus oryzae*.

Table 1. Analysis of aflatoxins B_1, B_2, G_1, G_2, ochratoxin A and cyclopiazonic acid in the culture material of *Aspergillus oryzae* after 10 days of cultivation.

Mycotoxin	LOD	Concentration in the culture material [1]
Aflatoxins B_1, B_2, G_1, G_2 (ng·g^{-1})	0.5	ND
Ochratoxin A (ng·g^{-1})	8.7	ND
Cyclopiazonic acid (µg·g^{-1})	5.0	14.5

[1]Mean value of 3 replicates analyzed; LOD, limit of detection; ND, Not detected in any sample analyzed.

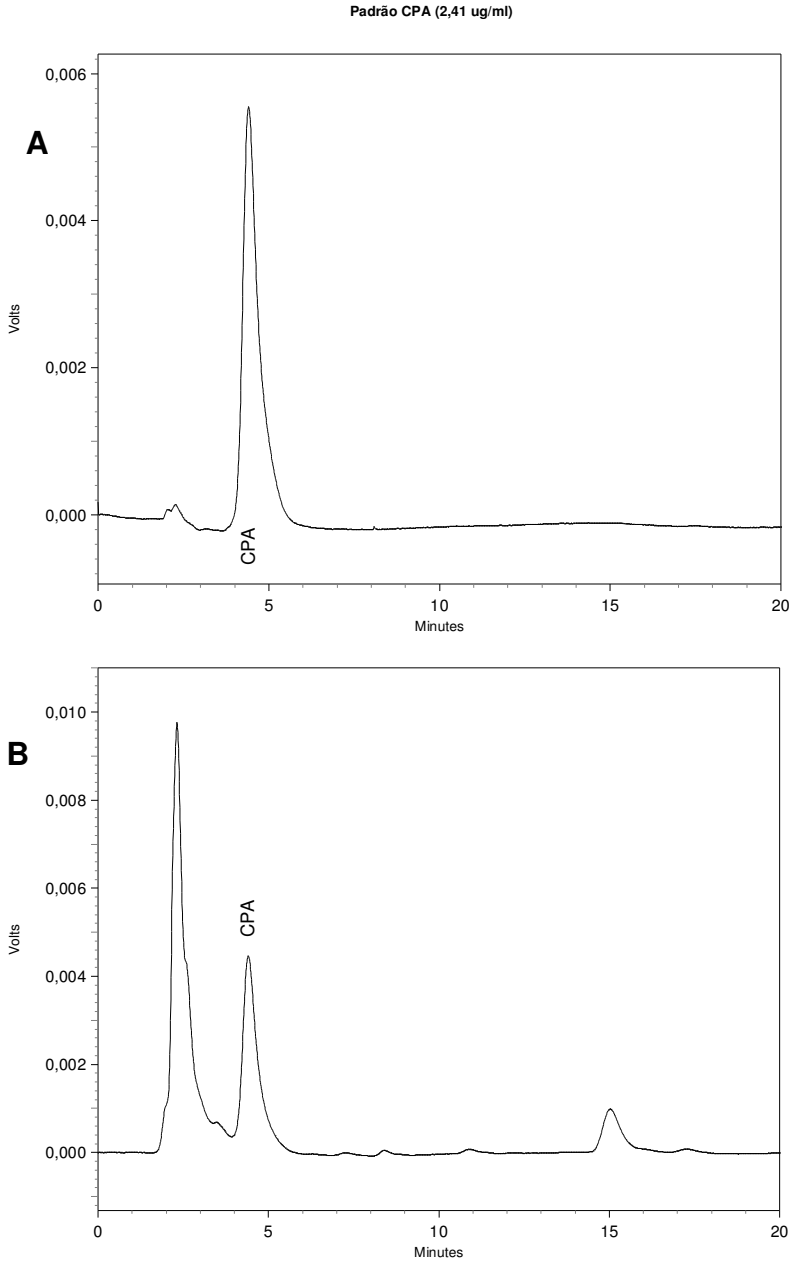

Figure 4. Chromatograms showing **(A)** Cyclopiazonic acid standard (2.41 $\mu g \cdot ml^{-1}$). **(B)** Sample of culture material of *Aspergillus oryzae* containing 12.0 $\mu g \cdot g^{-1}$

et al., 1994; Atalla et al., 2003). However, it is possible that these aflatoxin-producing strains were incorrectly identified as *A. oryzae* because of the close taxonomic relatedness between this fungus and other members of *A. flavus* group (Blumenthal, 2004).

The absence of OA in our study agree with those observed by Lane et al. (1997), who also didn't find this toxin in various batches of the enzyme preparations. Ochratoxins were first known to be produced by species from Section Circumdati (*A. orchraceus* group) (Cole and Cox, 1981). Moreover, the production of this toxin was repeatedly reported in Section Nigri (Martínez-Culebras et al., 2009), which stress the importance of testing the ochratoxin production in *Aspergillus*-derived enzyme preparations. All samples showed spots corresponding to the CPA standard onto TLC plates (Figure 1C), which were thereafter confirmed and quantified by HPLC. The mean concentration of CPA in the samples was 14.5 $\mu g \cdot g^{-1}$ (Figure 4). Production of CPA by *A. oryzae* was firstly reported by Orth (1977), and subsequently by other authors (Vinokurova et al., 2007). Although the molecular genetics of CPA biosynthesis have not been as well studied as for aflatoxins

biosynthesis, some essential genes for the toxin pathway in the CPA-producing *A. oryzae* have been identified (Christensen et al., 2005; Tokuoka et al., 2008). It is presumed that some chromosomal deletion that affects aflatoxin production *A. oryzae* strains can also affects CPA production (Tokuoka et al., 2008; Chang et al., 2009). On the other hand, only a certain group of aflatoxin-nonproducing strains have the potential to produce CPA (Kusumoto et al., 2000; Tokuoka et al., 2008). According to the results obtained in our study, the evaluated strain seems to belong to this group. Production of CPA in *A. oryzae* was reported to start nearly 50 h after inoculation, reaching maximum levels in two weeks (Goto et al., 1987). Fondevila et al. (1990) reported that fermentation extracts of *A. oryzae* extracts remains in the rumen for less than 24 h. There-fore, when using *A. oryzae* as feed additive for ruminants, the short duration of the fungus in the rumen would not be enough to allow the production of secondary metabolites such as CPA and cause toxic effects.

Conclusions

The *A. oryzae* strain tested does not produce detectable levels of aflatoxins B_1, B_2, G_1 or G_2, and ochratoxin A. Although cyclopiazonic acid was detected in the fungal extracts during 10 days of cultivation, the amount produced is of negligible toxicology significance for animal health, taking into account the short period of the fungus in the rumen. The *A. oryzae* strain tested therefore could be used as a feed additive for ruminants.

REFERENCES

Adebajo LO (1992). Spoilage moulds and aflatoxins from poultry feeds. Food/Nahrung 36:523-529.

Atalla MM, Hassanein NM, El-Beih AA, Youssef YA (2003). Mycotoxin production in wheat grains by different *aspergillius* in relation to different relative humidities and storage periods. Food/Nahrung 47:6-10.

Beuchat LR (2001). Traditional fermented foods. In: Doyle MP, Beuchat LR, Montville TJ (Eds.), Food Microbiology Fundamentals and Frontiers, 2nd ed. ASM Press, Washington DC, pp.701-719.

Blumenthal CZ (2004). Production of toxic metabolites in *Aspergillus niger, Aspergillus oryzae*, and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regul. Toxicol. Pharmacol. 39:214-228.

Chang PK, Horn BW, Dorner JW (2009). Clustered genes involved in cyclopiazonic acid production are next to the aflatoxin biosynthesis gene cluster in *Aspergillus flavus*. Fungal Gen. Biol. 46:176-182.

Christensen BE, Mollgaard H, Kaasgaard S, Lehmbeck J (2005). Methods for producing polypeptides in *Aspergillus* mutant cells. United States Patent 20050153397.

Cole RJ, Cox RH (1981). Handbook of Toxic Fungal Metabolites. Academic Press, New York.

Di Francia A, Masucci F, De Rosa G, Varricchio ML, Proto V (2008). Effects of *Aspergillus oryzae* extract and a *Saccharomyces cerevisiae* fermentation product on intake, body weight gain and digestibility in buffalo calves. Anim. Feed Sci. Technol. 140:67-77.

El-Kady I, El-Maraghy S, Zohri A (1994). Mycotoxin producing potential of some isolates of *Aspergillus flavus* and Eurotium groups from meat products. J. Microb. Res. 149:297-307.

Fondevila M, Newbold CJ, Hotten PM, Orskov ER (1990). A note on the effect of *Aspergillus oryzae* fermentation extract on the rumen fermentation of sheep given straw. Anim. Prod. 51:422-425.

Food and Agriculture Organization (2001). Summary and conclusions of the Joint FAO/WHO Expert Committee on Food Additives. FAO, Rome. (JECFA 57th meeting).

Gómez-Alarcón RA, Huber JT, Higginbotham GE, Wiersma F, Ammon D, Taylor B (1991). Influence of feeding *Aspergillus oryzae* fermentation extract on the milk yields, eating patterns and body temperatures of lactating cows. J. Anim. Sci. 69:1733-1740.

Goto T, Shinshi E, Tanaka K, Masaru M (1987). Production of cyclopiazonic acid by koji molds and possibility of cyclopiazonic acid contamination of Japanese fermented foods. Shokuhin Sogo Kenkyusho Kenkyu Hokoku 51:23-28.

Humphry JB, Coffey KP, Moyer JL, Brazle FK, Lomas LW (2002). Intake, digestion, and digestive characteristics of Neotyphodium coenophialum-infected and uninfected fescue by heifers offered hay diets supplemented with *Aspergillus oryzae* fermentation extract or laidlomycin propionate. J. Anim. Sci. 80:225-234.

Kamra DN, Agawal N (2004). Probiotics as feed additives for the ruminants. Indian Vet. Res. Inst. Izatnagar, India. 43:122-127.

Kim HS, Ahn BS, Chung SG, Moon YH, Ha JK, Seo IJ, Ahn BH, Lee SS (2006). Effect of yeast culture, fungal fermentation extract and non-ionic surfactant on performance of Holstein cows during transition period. Anim. Feed Sci. Technol. 126:23-29.

Kiyota T, Hamada R, Sakamoto K, Iwashita K, Yamada O, Mikami S (2011). Aflatoxin non-productivity of Aspergillus oryzae caused by loss of function in the aflJ gene product. J. Biosci. Bioeng. 111:512-517.

Klich MA, Pitt JI (1988). A laboratory guide to common Aspergillus species and their teleomorphs. CSIRO, North Ryde, NSW, Australia.

Kusumoto KI, Goto T, Manabe M (1990). Evaluation for conversion of sterigmatocystin to aflatoxin in koji-molds. Rep. Natl. Food Res. Inst. 54:14-17.

Kusumoto KI, Nogata Y, Ohta H (2000). Directed deletions in theaflatoxin biosynthesis gene homolog cluster of *Aspergillus oryzae*. Curr. Genet. 37:104-111.

Lane RW, Yamakoshi J, Kikuchi M, Mizusawa K, Henderson L, Smith M (1997). Safety evaluation of tannase enzyme preparation derived from *Aspergillus oryzae*. Food Chem. Toxicol. 35:207-212.

Lansden JA (1986). Determination of cyclopiazonic acid in peanuts and corn by thin layer chromatography. J. Assoc. Off. Anal. Chem. 69:964-966.

López-Díaz TM, Santos JA, García-López ML, Otero A (2001). Surface mycoflora of a Spanish fermented meat sausage and toxigenicity of Penicillium isolates. Int. J. Food Microb. 68:69-74.

Martínez-Culebras PV, Crespo-Sempere A, Sánchez-Hervás M, Elizaquivel P, Aznar R, Ramón D (2009). Molecular characterization of the black *Aspergillus* isolates responsible for ochratoxin A contamination in grapes and wine in relation to taxonomy of *Aspergillus* section Nigri. Int. J. Food Microb. 132:33-41.

Murakami H (1971). Classification of the koji mold. J. Gen. Appl. Microb. 17:281-309.

Orth R (1977). Mycotoxins of *Aspergillus oryzae* strains for use in the food industry as starters and enzyme producing molds. Ann. Nutr. Alim. 31:617-624.

Pariza MW, Johnson EA (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Reg. Toxicol. Pharmacol. 33:173-186.

Reddy TV, Viswanathan L, Venkitasubramanian TA (1979). Factors affecting aflatoxin production by *Aspergillus parasiticus* in a chemically defined medium. J. Gen. Microbiol. 114:409-413.

Soares LMV, Rodrigues-Amaya DB (1989). Survey of aflatoxins, ochratoxins A zearalenona and sterigmatocystin in some Brazilian foods by using multitoxin thin layer chromatografic method. J. Assoc. Off. Anal. Chem. 72:22-26.

Tokuoka M, Seshime Y, Fujii I, Kitamoto K, Takahashi T, Koyama Y (2008). Identification of a novel polypeptide synthase-nonribosomal peptide synthetase (PKS-NRPS) gene required for the biosynthesis of cyclopiazonic acid in Aspergillus oryzae. Fungal Genet. Biol. 45:1608-1615.

Urano T, Trucksess MW, Matusik J (1992). Liquid chromatographic

determination of cyclopiazonic acid in corn and peanuts. J. Assoc. Off. Anal. Chem. 75:319-322.
Vinokurova NG, Ivanushkina NE, Khmel'nitskaya II, Arindabarov MU (2007). Synthesis of a-cyclopiazonic acid by fungi of the genus Aspergillus. Appl. Biochem. Microb. 43:435-438.
Wiedmeier RD, Arambel MJ, Walters JL (1987). Effect of yeast culture and *Aspergillus oryzae* fermentation extract on ruminal characteristics and nutrient digestibility. J. Dairy Sci. 70:2063-2068.