

## Identification of toxigenic *Aspergillus* species from diet dairy goat using a polyphasic approach

### Identificação de espécies toxigênicas de *Aspergillus* isoladas da dieta de cabras leiteiras utilizando uma abordagem polifásica

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#### ABSTRACT

Some species of filamentous fungi that infest agricultural commodities are able to produce mycotoxins, contaminating feed and animal products. The aim of this research was to identify the mycoflora present in the feed and forage for dairy goat and to isolate and characterize the *Aspergillus flavus* and *A. parasiticus* strains based on a morphological and molecular characterization and mycotoxigenic ability. The goat dairy diets were collected monthly from 11 goat milk farms, totaling 129 and 106 samples of concentrate and forage, respectively. For the isolation of the mycobiota the surface plating method was used. *Aspergillus*, *Penicillium*, and *Fusarium* were the main fungi producing mycotoxins isolated. The morphological and molecular characterization and mycotoxigenic ability were used for *A. flavus* and *A. parasiticus* identification. The *Aspergillus* spp. from feed 39% produced aflatoxins B<sub>1</sub> and B<sub>2</sub>, 17% produced cyclopiazonic acid (CPA), 18% produced both toxins, and 42% had no toxigenic ability. Only 2.0% of the strains produced aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>, but no CPA. The strains from forage were producers of aflatoxins B<sub>1</sub> and B<sub>2</sub> (37%), CPA (14%), 14% of both mycotoxins, whereas 49% have shown no toxigenic ability. The *aflD* and *aflR* genes were used by PCR and PCR-RFLP, respectively. The presence of toxigenic species in samples of feed for lactating goats indicates a potential risk of contamination of dairy products, if they are exposed to environmental conditions favorable to fungal growth and mycotoxin production.

**Key words:** toxigenic fungi, aflatoxin, cyclopiazonic acid, feed, molecular characterization.

#### RESUMO

Algumas espécies de fungos filamentosos que infestam os produtos agrícolas e ração são capazes de produzir micotoxinas. O objetivo deste trabalho foi identificar a micoflora presente nos concentrados e volumosos utilizados na dieta de cabras leiteiras e isolar as espécies *Aspergillus flavus* e *A.*

*parasiticus*, com base em uma caracterização morfológica e molecular e capacidade micotoxigênica. Os alimentos foram coletados mensalmente em 11 fazendas produtoras de leite de cabra, totalizando 129 e 106 amostras de concentrado e volumoso, respectivamente. Para o isolamento da micobiota, foi utilizado o método de plaqueamento de superfície. *Aspergillus*, *Penicillium* e *Fusarium* foram os principais gêneros de fungos produtores de micotoxinas isolados das amostras. A caracterização morfológica e molecular e capacidade micotoxigênica foram utilizadas para identificação de *A. flavus* e *A. parasiticus*. Das cepas *Aspergillus* spp isoladas do concentrado, 39% produziram aflatoxinas B<sub>1</sub> e B<sub>2</sub>, 17% produziram ácido ciclopiazônico (ACP), 18% produziram ambas as toxinas e 42% não tinham capacidade toxigênica. Apenas 2,0% das cepas produziram aflatoxinas B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> e G<sub>2</sub>. As cepas de *Aspergillus* spp. isoladas do volumoso foram produtores de aflatoxinas B<sub>1</sub> e B<sub>2</sub> (37%), ACP (14%), sendo que 14% produziram ambas as toxinas e 49% não foram produtoras. Os genes *aflD* e *aflR* foram utilizados para a PCR e a PCR-RFLP, respectivamente. A presença de espécies toxigênicas em amostras de alimentos destinados a caprinos em lactação indica um risco potencial de contaminação dos produtos lácteos por aflatoxinas e ACP, caso estes sejam expostos a condições ambientais favoráveis ao crescimento de fungos e produção de micotoxinas.

**Palavras-chave:** fungos toxigênicos, aflatoxinas, ácido ciclopiazônico, ração, caracterização molecular.

#### INTRODUCTION

The complex diet of ruminants, consisting of forages, concentrates, and silages, can be a source of diverse mixture of mycotoxins that contaminate individual feed components (SMITH & KOROSTEVA, 2012). Both, feed grains (e.g.

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corn, wheat, barley, cottonseed) and forages support fungal growth that leads to mycotoxin production (OSWEILER, 2012). MORETTI et al. (2013) emphasize the importance of knowledge of biodiversity of toxigenic fungi to better understand factors that contribute to mycotoxin production, assessment of risks posed by mycotoxigenic fungi, and reduction of mycotoxin contamination in feed and food crops. The mycotoxigenic fungi involved in the food and feed chain belong mainly to the *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* genera. *Aspergillus* section Flavi includes three species, *A. flavus*, *A. parasiticus* and *A. nomius*, producers of aflatoxins, highly toxic compounds of concern in food safety. *A. flavus* also produces other mycotoxin such as cyclopiazonic acid (CPA) (VAAMONDE et al., 2003). The identification of *Aspergillus* Section Flavi has been traditionally based on morphological and biochemical characterization. Conidial wall ornamentation is regarded as the primary morphological diagnostic character for separation of *Aspergillus flavus* and *A. parasiticus*. The mycotoxigenic profile (regarding aflatoxins B and G and CPA) of these strains has been routinely used for identification (RODRIGUES et al., 2009). Molecular techniques have been used to differentiate these two species (SOMASHEKAR et al., 2004; RODRIGUES et al., 2009). However, it is to be emphasized that the PCR detection of *A. flavus*, *A. parasiticus* or *A. nomius* is no guarantee of aflatoxin production since gene other than those involved in the biosynthesis of aflatoxins are not target for amplification (LEVIN, 2012). The State of São Paulo has environmental factors conducive to the consolidation of goat and sheep production, and the scientific and technological advances contribute to increased rates of production and productivity of animals. Since the aflatoxins and CPA can be toxic compounds of concern in milk, it is very important to offer a quality feed for dairy goats. So, the aim of this work was to identify the mycoflora present in the feed and forage for dairy goat and isolate and characterize the *A. flavus* and *A. parasiticus* strains based on a polyphasic approach involving morphological and molecular characterization, and mycotoxigenic ability.

## MATERIAL AND METHODS

### Samples

The samples of dairy goat feed (129) and forage (106) were collected from May, 2010 to September, 2011, once a month from 11 farms producing goat milk located in the cities of Ibiúna, Piedade, Alambari, Capão Bonito, Guareí, and Porto Feliz, in São Paulo State, Brazil. In the properties,

feed and forage were packed in barrels with a capacity of 20 kg each. Samples of the feed and forage were collected at nine different points of each barrel: three points of the upper third, three points of the middle third and three points of the lower third (SASSAHARA et al., 2003). Each sample contained a minimum of 500 g. The collected material was homogenized and stored in plastic packaging and sent to the laboratory for isolation and identification of the mycoflora and water activity analysis.

### Identification and enumeration of the mycobiota from goat feed and forage

The animal feed and forage samples were apportioned in 10g aliquots and homogenized for 30 minutes in bottles containing 90mL of sterile distilled water. Aliquots with 0.1mL of the dilutions in serial from  $10^{-1}$  to  $10^{-6}$  of the samples were plates in duplicate using the surface methods in potato agar dextrose (PDA) medium. The plates were incubated at 25°C for 5 days, but the observations were made daily. The colonies were identified at the genus level, and those belonging to the genus *Aspergillus* were identified at the species level according to PITT & HOCKING (1997).

### Molecular Characterization of *A. flavus* and *A. parasiticus*

#### DNA extraction

The strains characterized as the genus *Aspergillus* (233) were submitted to molecular identification. The strains of *A. flavus* and *A. parasiticus* were maintained in a tube containing PDA at 25°C at 7 days, and a loop full of spores from each strain was transferred to 1.5mL of lysis buffer with 1.0g of sterile acid-washed 0.4 to 0.6mm diameter glass beads and vortexed for 5.0min at maximum speed. Proteins and polysaccharides were precipitated by 750µL of cold 3M sodium acetate, pH 5.5. The solution was mixed, placed at -20°C for 10min and centrifuged at 5000g for 10min (4°C) for twice. The supernatant was precipitated with one volume of cold isopropanol. The solution was mixed gently, incubated for 1.0h at -20°C and centrifuged at 5000g for 10min at 4°C. DNA pellet was washed twice with 1.0mL of cold 70% ethanol, centrifuged at 5000g for 5min at 4°C. DNA was diluted in 100µL of ultrapure water and stored at -20°C (RODRIGUES et al., 2009). The DNA was quantified by measuring the absorption at 260nm in a Hitachi U-2000 spectrophotometer.

#### PCR amplification of *afID* gene

The gene *afID* was tested for all strains and the *afID* primers were specifically designed according

to the described by RODRIGUES et al. (2009). The PCR amplifications were performed using about 1 µg of fungal template DNA, 200 pmol of each primer (*Nor1*-Forward: 5'-ACC GCT ACG CCG GCA CTC TCG GCA C-3' and *Nor2*-Reverse: 5'-GTT GGC CGC CAG CTT CGA CAC TCC G-3'), MgCl<sub>2</sub>-free reaction buffer, 2.0mM MgCl<sub>2</sub>, 2.5U of Taq polymerase and 0.2mM of each dNTP. PCR was carried out under the following conditions: one cycle at 94°C for 3min; 30 cycles at 94°C for 1min, at 55°C for 1min and at 72°C for 1min; and at 72°C for 10min in a final extension.

#### Restriction site analysis of PCR products

After analysis of PCR products by amplification of *aflD* gene, only the samples positive for *Aspergillus* fungi were used in the restriction site analysis. First, a new PCR was used to amplify two target fragments on *A. flavus* and *A. parasiticus*, and the *aflR* primer sequences were designed according to the described by SOMASHEKAR et al. (2004) to amplify a fragment of 796pb (*aflR1*-Forward: 5'-AAC CGC ATC CAC AAT CTC AT-3' and *aflR2*-Reverse: 5'-AGT GCA GTT CGC TCA GAA CA-3'). The reaction mixture consisted of 1.0µg of fungal template DNA, 50pmol of each primer, MgCl<sub>2</sub>-free reaction buffer, 2.0mM MgCl<sub>2</sub>, 0.5U of Taq polymerase and 0.2mM of each dNTP. PCR was carried out under the following conditions: one cycle at 94°C for 5min; 30 cycles at 94°C for 3min, at 50°C for 1.25min and at 72°C for 1.40min; and at 72°C for 10min in a final extension. Then, the PCR products were digested with *HincII* to analyze the restriction site. Each restriction reaction contained 10µL of PCR product (about 50µg DNA), 10 units of *HincII* enzyme and 2.0µL of 10x digestion buffer as recommended by the manufacturer. The reaction mixture was incubated at 37°C for 4h. The sample was frozen and dried in nitrogen gas. After, the precipitate was dissolved in 10µL of water, and the resulting fragments were separated by electrophoresis on a 1.5% agarose gel. The primers were synthesized by Bioneer Corporation, South Korea, and all other reagents used in PCR and restriction analysis were purchased by Fermentas, Lithuania, part of Thermo Fisher Scientific.

#### Toxicogenic potential of *A. flavus* and *A. parasiticus* strains for aflatoxins

The production of aflatoxins was tested using the method of LIN & DIANESE, 1976. The *A. flavus* strains isolated from goat feed (130) and from forage (70) were maintained in a tube containing PDA at 25°C for 5 days. A fragment of each colony was then inoculated into a plate containing coconut

agar medium and incubated at 25°C for 10 days. After growth, the whole content of each plate was removed, and chloroform was added, 30mL for each 10g of culture. After shaking, the sample was filtered through filter paper with diatomaceous earth and sodium sulfate. The filtrate was evaporated, and the extract was suspended in chloroform and then submitted to thin layer chromatography (TLC).

#### Aflatoxin analyses

The aflatoxins identification and quantification were performed by TLC using an aliquot (40µL) of each sample, which was spotted on silica gel-G thin layer plate (Merck, Germany) and then developed with chloroform:acetone 9:1 (v/v) as a solvent system. The concentration of aflatoxins was determined by photodensitometry (Shimadzu, CS 9000) comparing the area and density of the spot samples with aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> standards (Sigma Aldrich, USA) (GONÇALEZ et al., 2001). The detection and quantification limits were established how the lowest fluorescence detectable signal and the lowest concentration measured, respectively. The detection and quantification limits for AFB<sub>1</sub> were 0.8ng g<sup>-1</sup> and 1.6ng g<sup>-1</sup>, respectively. Recovery for standard aflatoxin B<sub>1</sub> should be at least 94%.

#### Toxicogenic potential of *A. flavus* strains for CPA

The methodology of GONÇALEZ et al. (2013) was employed to evaluate the CPA production by *A. flavus* strains. The *A. flavus* strains isolated from goat feed (130) and from forage (70) were maintained in a tube containing PDA at 25°C for 7 days. Spore solutions (1.0mL) of each sample were inoculated into 25mL of Czapek-Dox broth (Difco) and incubated for 12 days at 25°C. The cultures were filtrated, and the CPA was extracted twice with 25mL of chloroform. The chloroform was evaporated and the extract was diluted in 1.0mL of methanol HPLC grade and then submitted to high performance liquid chromatography (HPLC).

#### HPLC conditions

The mobile phase consisted of methanol: water/zinc sulfate 4.0mM 7:3 (v/v) at a flow rate of 0.6mL min<sup>-1</sup>. A C<sub>18</sub> column, 250mm x 4.6mm (Shimadzu, Japan) was used, and the analyses were done with a UV detector at 284nm. The calibration curve was established by the external standard method with five concentrations: 0.611; 1.223; 2.446; 4.892, and 9.798µg mL<sup>-1</sup> (r<sup>2</sup>=0.998). The HPLC quantification and detection limits for a standard CPA were 0.030µg mL<sup>-1</sup> and 0.005µg mL<sup>-1</sup>, respectively. Recovery for standard CPA was 97%.

#### Water activity determination

The water activity ( $a_w$ ) of goat feed and forage samples was determined by automatic analysis using Aqualab 4TE (Decagon Devices Inc., Pullman, WA).

#### Statistical analysis

The results were analyzed by ANOVA and Tukey's multiple range tests with a significance level  $P < 0.05$  to determine differences between feed and forage using the variables: fungi isolation, *A. flavus* and *A. parasiticus* identification, mycotoxigenic ability and  $a_w$ .

## RESULTS AND DISCUSSION

An important component of efforts to control mycotoxin contamination problems is the study of the morphological, molecular genetics, metabolic and plant pathological diversity of mycotoxigenic fungi (MORETTI et al., 2013).

The mycoflora of the animal feed and forage samples are shown in table 1. Toxigenic fungi belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium* were isolated from most samples (Table 1). Other studies also reported the presence of *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., and *Eurotium* spp. in animal feed (PEREYRA et al., 2010; ASTORECA et al., 2011). Among representatives of the genus *Aspergillus*, were isolated from animal feed: *A. flavus* (69%), *A. fumigatus* (2.30%), *A. niger* (1.50%) and *A. parasiticus* (0.80%). The highest incidence of *A. flavus* was shown in animal feed and in their ingredients (PEREYRA et al., 2010 and 2011; ASTORECA et al., 2011). The *Aspergillus* species isolated from forage

were: *A. flavus* (49%), *A. fumigatus* (5.70%), and *A. niger* (1.0%). The forage samples showed highest incidence of *Aspergillus* spp. than in feed. Same results were found by ACCENSI et al. (2004). The identification of *Aspergillus* Section Flavi has been traditionally based on morphological and biochemical characterization (RODRIGUES et al., 2009).

The morphological and molecular characterization and mycotoxigenic ability were used for *A. flavus* and *A. parasiticus* identification. Morphologically, *A. flavus* has finely roughened conidia mostly produced from heads bearing both metulae and phialides, whereas conidia of *A. parasiticus* are usually conspicuously roughened and most heads bear phialides alone (PITT & HOCKING, 1997). Based on these morphological characters, it was possible to identify *A. flavus* in 64% of the *Aspergillus* spp. isolated from feed and 37% from the forage. The identification was confirmed by molecular characterization and mycotoxigenic ability of the *A. flavus* and *A. parasiticus* strains.

The results of the molecular analysis showed that 97% of the *Aspergillus* spp. isolated from feed was PCR positive for *A. flavus* and *A. parasiticus*. The molecular identification using the gene *afID* (PCR) was a good maker for differentiating the *A. flavus* and *A. parasiticus* species (fragment of 400pb) from the other *Aspergillus* spp., but it was not possible to differentiate them from each other. Of 131 strains isolated from feed and confirmed by PCR, 65.60% were *A. flavus* and 0.76% was *A. parasiticus*, using the gene *afIR* (PCR-RFLP). Among the *Aspergillus* spp. isolated from forage (98 strains) 71.40% were positive by PCR analysis and 51.40% of them were identified with *A. flavus*, however *A. parasiticus* was not isolated. The results obtained in this work are in agreement with SOMASHEKAR et al. (2004), who showed that PCR-RFLP patterns obtained with *HincII* can be used to distinguish the two species. *A. flavus* cleaved into 3 fragments of 385, 250, 161bp whereas *A. parasiticus*, having one restriction site for the *HincII*, produced 2 fragments of 546 and 250bp. The *afID* and *afIR* genes were not able to identify the ability of the strains to produce aflatoxins (SOMASHEKAR et al., 2004; RODRIGUES et al., 2009). In addition, the genomic structural genes involved in aflatoxins biosynthesis do not guarantee the production of aflatoxins by all isolates of *A. flavus* and *A. parasiticus* (LEVIN, 2012), so their mycotoxigenic ability was investigated. The 131 *Aspergillus* spp. strains confirmed by PCR from feed and 70 strains from the forage were evaluated as having ability to produce aflatoxins and CPA (Table 2). *A. flavus* is able to produce aflatoxins

Table 1 - Relative frequency (%) of the genera fungi contamination of animal feed (129 samples) and forage (106 samples) collected in 11 dairy goat farms in the period May, 2010 to September, 2011.

Isolated fungi	Relative frequency (%) in animal feed	Relative frequency (%) in forage
<i>Penicillium</i> spp.	84.5	71.7
<i>Mucor</i> spp.	44.9	30.2
<i>Fusarium</i> spp.	35.6	32.0
<i>Aspergillus</i> spp.	34.1	43.4
<i>Cladosporium</i> spp.	33.3	34.9
NSF*	31.0	50.0
<i>Rhizopus</i> spp.	21.0	8.5
<i>Trichoderma</i> spp.	20.9	16.0
<i>Colletotrichum</i> spp.	8.5	26.4
<i>Eurotium</i> spp.	1.5	2.3
<i>Paecilomyces</i> spp.	0.8	-

\*NSF: Non-sporulating fungi.

Table 2 - Aflatoxins B<sub>1</sub> and B<sub>2</sub> and CPA production in culture medium by *Aspergillus* spp. isolated from feed (131 strains) and forage (70 strains) collected in 11 dairy goat farms in the period May, 2010 to September, 2011.

Mycotoxin	----- Feed -----		----- Forage -----	
	Number of isolates producer (%)	Range	Number of isolates producer (%)	Range
AFB <sub>1</sub>	51 (39)	9.12 - 6,425.43µg kg <sup>-1</sup>	26 (37)	8.35 - 2,944.74µg kg <sup>-1</sup>
AFB <sub>2</sub>	16 (12)	7.56 - 87.18µg kg <sup>-1</sup>	05 (7)	3.57 - 49.24µg kg <sup>-1</sup>
CPA	22 (17)	2.12 - 179.06µg mL <sup>-1</sup>	10 (14)	4.10 - 123.92µg mL <sup>-1</sup>

AFB<sub>1</sub>: aflatoxin B<sub>1</sub>; AFB<sub>2</sub>: aflatoxin B<sub>2</sub>; CPA: cyclopiazonic acid.

and CPA, and *A. parasiticus* can produce only aflatoxins. The results showed that of the *Aspergillus* spp. from feed 39% produced aflatoxins B<sub>1</sub> and B<sub>2</sub>, 17% produced CPA, 18% produced both toxins, and 42% had no toxigenic ability. Only 2.0% of the strains produced aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> but no CPA, and, based on all characteristics analyzed, they were identified with *A. parasiticus*. Almost all *A. parasiticus* isolated produce both aflatoxins B and G, but not CPA (HORN & DORNER, 1999). The strains from forage were producers of aflatoxins B<sub>1</sub> and B<sub>2</sub> (37%), CPA (14%), 14% of both mycotoxins, and 49% did not show toxigenic ability. The concentrations of aflatoxins B<sub>1</sub> and B<sub>2</sub> and CPA were showed in the table 2. The results showed the great variability in the mycotoxins producing potential by *A. flavus* in the culture conditions. Our results agree with the literature that also found difference in the mycotoxigenic ability of the *A. flavus* (VAAMONDE et al., 2003; ASTORECA et al., 2011). VAAMONDE et al. (2003) proposed five chemotypes for *A. flavus* species, based on the mycotoxins produced by them: chemotype I for aflatoxin B and CPA producers; II for AFB, AFG and CPA producers; III for AFB producers; IV for CPA producers and V for non mycotoxin producers. The *A. flavus* strains isolated from feed and forage belong to the following chemotypes, respectively: type I (18% and 14%); type III (39% and 37%); type IV (17% and 14%) and type V (25% and 35%). The chemotype II was not isolated. ASTORECA et al. (2011), also isolated *A. flavus* belonging to chemotypes I, III, IV and V, but no type II from poultry feed. By comparing the chemotypes isolated from feed and forage, there was no statically significant difference between them (P<0.05).

The average a<sub>w</sub> values of the samples ranged from 0.51 to 0.75 for feed and from 0.46 to 1.0 for forage. The minimum a<sub>w</sub> values required for growing *A. flavus* and *A. parasiticus* as well as for producing aflatoxins are 0.80 and 0.83, respectively (PITT & HOCKING, 1997). The a<sub>w</sub> did not influence the *A. flavus* and *A. parasiticus* isolation from feed

and also forage (P<0.05) since these species were isolated in all samples analyses. Therefore, the feed did not have water enough for the fungal activity. On the other hand, the forage is not a good substrate, because it has high fiber concentration and low nutrients to the fungi.

According to SMITH & MOSS (1985), feed with good microbiological quality should present a maximum of 10<sup>5</sup> colony-forming units g<sup>-1</sup> (CFU g<sup>-1</sup>). The results showed that only 17% of the feed and 2.8% of forage samples had more than 10<sup>5</sup>CFU g<sup>-1</sup>, therefore most of goat feeding had good quality in all farms studied.

## CONCLUSION

The results obtained in this study indicate that, to distinguish the *A. flavus* and *A. parasiticus* species, it is necessary more than one technique of identification. The presence of *A. flavus* capable of producing CPA and aflatoxins indicates a risk of contamination in dairy goat feed and forage if they are exposed to environmental conditions that allow fungal growth.

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