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Toxigenic fungi and mycotoxins in mature corn silage

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Abstract

To investigate the exposure of livestock and farm workers to mycotoxins during the last months of silage use, the mycoflora and the mycotoxins in a mature silage (11-months-old) were studied. A multimycotoxin method was developed to evaluate the toxigenic *in vitro* ability of fungal strains. The screening of potentially toxigenic fungi isolated from the mature silage showed that six *Fusaria* (*Fusarium culmorum*, *Fusarium equiseti*, *Fusarium graminearum*, *Fusarium oxysporum*, *Fusarium solani* and *Fusarium verticillioides*) and one *Aspergillus* (*Aspergillus fumigatus*) were able to produce mycotoxins on nutrient agar. Seven major mycotoxins (aflatoxin B_1, citrinin, deoxynivalenol, fumonisin B_1, gliotoxin, ochratoxin A and zearalenone) were also searched in the corn silage by high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS). Among the three mycotoxins (citrinin, gliotoxin and deoxynivalenol) detected in the silage, gliotoxin, a strongly immunosuppressive mycotoxin, occured in the mature silage at level up to 877 ppb, which was associated with the presence of *A. fumigatus* in the silage.

Keywords: Mature corn silage; Mycoflora; Multimycotoxin; HPLC-MS

1. Introduction

Corn silage (*Zea mays* L.) is one of the most important cattle feeds in France (AGRESTE, 2000). Its production was essentially based on the principle of preservation under anaerobic conditions together with the growth of lactic acid bacteria which promote a natural fermentation, lowering the pH to a level at which clostridial growth is prohibited. These conditions (low pH and anaerobiosis) were considered as unfavourable for the growth of most molds. However, this matrix could be exposed to fungal development, particularly at the end of corn silage use. The presence of microfungi could be attributed to the infection of corn at the pre-harvest stage (in the field) and/or at the post-harvest stage (during silage storage). Among these toxigenic species, *Aspergillus flavus* and *Fusarium verticillioides* were able to produce mycotoxins on corn (Cleveland et al., 2003).

The fungal growth reduced nutritional value and could result in the production of mycotoxins (Frisvad et al., 2006) and allergenic spores (Adhikari et al., 2004) that constitute a risk factor for human and animal health. Toxic syndromes caused by mycotoxin ingestion were indicated as mycotoxicosis. Surveillance for mycotoxins like aflatoxins, ochratoxin A, zearalenone and fumonisins in cereals and animal feeds has shown that, where mycotoxins were identified, mixtures of these toxins often occurred (Scudamore et al., 1998). The daily handling of silage spoiled by fungal toxins could be a potential risk factor for human or animal safety. These observations showed the importance of developing multimycotoxin analysis methods as tools for the exposure studies.

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Few data were available on the fungal contamination and the ability of local strains to produce mycotoxins in corn silage, particularly in a mature silage predictably exposed to factors promoting mold proliferation and mycotoxin production such as oxygen, heat, rain or insect damage.

The aims of this multimycotoxin report were: (i) to investigate the mycoflora of a mature corn silage (11-months-old), (ii) to develop a multimycotoxin method used to assess the toxigenic *in vitro* ability of the fungal species, (iii) to compare the concentrations of seven mycotoxins at two levels in the corn silage (top and bottom).

2. Materials and methods

2.1. Chemicals and reagents

Seven mycotoxin standards (aflatoxin B_1, citrinin, deoxynivalenol, fumonisin B_1, gliotoxin, ochratoxin A and zearalenone) were supplied by Sigma–Aldrich (St. Louis, MO, USA). The stock solutions of 250 μg/ml were prepared in LC grade methanol from Chromanorm VWR Prolabo (Fontenay-sous-Bois, France) and stored at −20 °C in the dark. Diluted solutions were prepared immediately before use by diluting the stock solutions with mobile phase acetonitrile/water (10:90, v/v). The working solution was composed of the seven mycotoxins. It was prepared by combining suitable aliquots of each individual standard stock dilution in order to obtain each mycotoxin at 1.25 μg/ml.

Milli-Q quality water (Millipore, Bedford, USA) and all other chemicals of LC grade were obtained from Prolabo.

Purification assays used Oasis HLB (6 ml, 200 mg) cartridges purchased from Waters (Milford, MA, USA).

2.2. Sample collection

Corn silage was located in a dairy farm of Normandy (France) characterized by 150 hectares of cultivated land with 21 hectares of corn and 205 cattle daily fed corn silage. Samples of a mature corn silage were collected at the end of the silage use (11-months-old).

Samples were taken through 15 cm in the "trench-type" silo at two levels (0.75 m from top and bottom of the silage) and the temperature was recorded. For each level, 800 g was mixed and ground in a blender in order to obtain an homogeneous sample. Hundred gram was then taken for the analysis of mycoflora and eight aliquots of 5 g were stored at −20 °C before their multimycotoxin analysis.

2.3. Mycological analysis

For each sample, 100 g of silage was suspended in 500 ml of sterile water containing sodium dodecyl sulfate (0.05%, w/v). After 1 h of magnetic shaking, 1 ml of each suspension was sprayed in a Petri dish (90 mm diameter) containing malt extract (1.5%)/agar (1.5%) medium (MEA) complemented with chloramphenicol (0.05%, w/v) following the soil plates method of Warcup (1950). To limit proliferous fungi like *Trichoderma* spp., MEA complemented with malachite green (0.0025%, w/v) was also used (Davet and Rouxel, 1997).

The plates were incubated at 24 and 37 °C. The identity of each strain, isolated and purified, was achieved through macro and microscopic examinations (Booth, 1966; Pitt, 1979; Domsch et al., 1980; Von Arx, 1981; Klich, 2002; Samson et al., 2002; Samson and Frisvad, 2004). For the species belonging to the *Penicillium* genus, growth was also observed on two selective media, Czapek yeast autolysate agar (CYA) and 25% glycerol nitrate agar (G25N), and incubated at 5, 22 and 37 °C (Pitt, 1979). *Fusarium* species were cultured on MEA and potato dextrose agar medium (PDA).

All the purified strains were preserved on agar slants (MEA) at 4 °C.

2.4. Multimycotoxin analysis by HPLC-MS

Liquid chromatography was performed using an Agilent Technologies series 1100 (Palo Alto, USA) quaternary pump coupled with an autosampler and a model SL mass spectrometric detector. The analytes were chromatographed at 40 °C on a 150 × 2.1 mm i.d., 5 μm, Zorbax SB-C_{18} column (Agilent Technologies, Palo Alto, USA) with a 1 mm Optiguard C_{18} precolumn. Mycotoxins were separated using gradient elution with acetonitrile as mobile phase A and water acidified with 0.5% acetic acid (pH 3) as mobile phase B. The gradient program was: at time zero, 5% solvent A; linear gradient to 50% solvent A within 15 min and to 80% at time 25 min. The flow rate was 300 μl/min. The injection volume was 25 μl. The retention times of mycotoxins were respectively 15.4, 16.7, 6.9, 15.1, 14.2, 20.3 and 20.4 min for aflatoxin B_1, citrinin, deoxynivalenol, fumonisin B_1, gliotoxin, ochratoxin A and zearalenone.

Mass spectrometry was performed on a quadrupole analyser equipped with electron spray ionization (ESI) source and operating in positive and negative modes. The parameters used for the mass spectrometer in all experiments were: capillary voltage 3.0 kV, solvent gas 720 l/h, evaporation temperature 350 °C and pressure of nebulization 35 psi. The ions monitored were 313, 335, 647 (aflatoxin B_1); 251, 233, 273 (citrinin); 265, 295, 297, 355 (deoxynivalenol); 722, 723, 724 (fumonisin B_1); 263 (gliotoxin); 404, 405, 426 (ochratoxin A); 317, 318, 319 (zearalenone). Full scan mass spectra were recorded in order to select the most abundant *m*/*z* value and then the selected ion monitoring mode (SIM) was used for the quantification.

2.5. Multimycotoxigenic in vitro ability of fungal strains

A multimycotoxin method was developed to simultaneously assess the ability of the fungal strains to produce seven major mycotoxins: aflatoxin B_1, citrinin, deoxynivalenol, fumonisin B_1, gliotoxin, ochratoxin A and zearalenone. For each dish, three agar plugs measuring 8 mm in diameter were removed from the central area of the colony (including conidia and mycelium), pooled, weighted and transferred to 5 ml glass vials. All mycotoxins were extracted by 2 ml of ethyl acetate acidified with 1% acetic acid except fumonisin B_1 which required 2 ml of methanol acidified with 1% acetic acid. After 15 min of centrifugation at 1500 rpm, each extract was evaporated to dryness under a stream of nitrogen. The final residue was dissolved in 0.5 ml of a mixture acetonitrile–water (10:90, v/v) and filtered through Millex HV 0.45 μm before the injection into the HPLC-MS. Analytical recoveries, carried out by spiking experiments, were 100% (aflatoxin B_1 and gliotoxin), 96% (deoxynivalenol), 86% (citrinin and ochratoxin A), 82% (zearalenone) and 65% (fumonisin B_1).

To evaluate the method, a screening of the potentially toxigenic fungal species isolated from the mature silage (Table 1) was conducted. These fungal strains were grown in triplicate on two different media, MEA and 1.5% corn steep medium (CS), a complex medium adapted from Cullen et al. (1982) during 2 weeks at 25 °C before their multimycotoxin analysis.

2.6. Determination of seven mycotoxins in corn silage

Mycotoxins were extracted and purified as previously described by Garon et al. (2006). The method has been optimized with the extraction and purification of fumonisin B_1 from corn silage. The clean-up step on Oasis HLB cartridge consisted in a double elution with 5 ml of pure methanol following by 5 ml of MTBE–methanol (90:10, v/v). After evaporation, the final residue was dissolved in 1 ml of mobile phase, filtered through Millex® HV 0.45 μm and injected into the HPLC-MS. The analytical recoveries were found to be, respectively, 65% (aflatoxin B_1, deoxynivalenol, ochratoxin A and zearalenone), 70% (gliotoxin), and 80% (citrinin and fumonisin B_1). The detection and quantification limits for aflatoxin B_1, citrinin, fumonisin B_1 and ochratoxin A were, respectively, 1.5 and 5 ppb, 6.5 and 20 ppb for deoxynivalenol, gliotoxin and zearalenone.

The flow chart of analytical procedure for the determination of mycotoxins in corn silage is summarized in Fig. 1.