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Eurotium spp. and Echinulin in Feed Refused by Swine

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Feed refused by swine contained a high-propagule density of *Eurotium chevalieri* Mangin (anamorph, *Aspergillus chevalieri* (Mangin) Thom and Church), *Eurotium amstelodami* Mangin (anamorph, *Aspergillus amstelodami* (Mangin Thom and Church), and *Aspergillus candidus* Link. Echinulin (8 μg/g of feed) was detected in the feed. Isolates of *E. chevalieri* and *E. amstelodami* but not *A. candidus* produced echinulin on rice or cracked corn. Mice refused to drink water containing 90 μg of echinulin per ml. This is the first report of the alkaloid echinulin in feed refused by swine.

A pelleted swine feed sample was received from the Veterinary Diagnostic Laboratory, University of Illinois, Urbana, in the autumn of 1986. The farmer who submitted the feed sample reported that boars and sows refused to eat the feed. Subsequently, the sow's milk production decreased and piglets died. Death of some sows also occurred. When a different ration was fed to the remaining boars and sows, they began to eat and their condition improved. Chemical analysis for traditional trichothecene refusal factors by the Animal Disease Laboratory, Centralia, Ill., revealed a low level of vomitoxin in the feed (0.3 μg/g). This level of vomitoxin would not account for the refusal activity observed (R. Lambert, unpublished data).

Examination of the swine feed with the aid of a dissecting microscope revealed abundant conidial structures typical of the *Aspergillus glaucus* group (8). The mycoflora of the feed sample were determined by dilution plating after separate blending of three 2-g subsamples with 198 ml of sterile water for 1 min at low speed. Dilutions were made from the resultant slurry and spread on plates of glucose-peptone-yeast extract agar (6). Plates were incubated at 25°C for 10 days. The total number of fungal colonies was determined, and representative colonies were subcultured for identification.

The feed sample contained 7×10^6 fungal propagules per g of feed, as determined by dilution plating. The species of prevalent molds recorded from the dilution plates were *Eurotium chevalieri* Mangin (anamorph, *Aspergillus chevalieri* (Mangin) Thom and Church), *Aspergillus candidus* Link, and *Eurotium amstelodami* Mangin (anamorph, *Aspergillus amstelodami* (Mangin) Thom and Church). They accounted for 66, 25, and 4% of the total propagule density, respectively. Representative isolates of each of these species were deposited in the Agricultural Research Culture Collection, Northern Regional Research Center, Peoria, Ill., as *E. chevalieri* NRRL A-27540, *E. amstelodami* NRRL A-27539, and *A. candidus* NRRL A-27538.

Each isolate was tested for refusal factor production by growth on cracked corn or rice; extracts were used in a mouse drinking water bioassay (1). Solid-state fermentations were carried out in 500-ml Erlenmeyer flasks containing 50 g of substrate. The moisture levels of the substrate were adjusted to 30 to 33% with distilled water before autoclaving. Each flask was inoculated with 1.0 ml of a spore suspension prepared by adding 5 ml of sterile distilled water to a 7-day-old potato glucose agar slant culture and agitating. Flasks were incubated at 25°C as static cultures for 2 weeks.

Refusal factors were extracted from the 50-g corn or rice cultures by blending with 250 ml of acetone for 3 min. The acetone extract was dried over anhydrous sodium sulfate, filtered by gravity, and evaporated to dryness, and the remaining residue was added to the drinking water of mice at a level of 100 ppm (100 μg/ml). Fifteen Harlan Sprague-Dawley (Institute for Cancer Research) female or male mice, 5 to 7 weeks old, were used in the drinking water bioassay. Residues from the acetone-extractable substances from the solid-state fermentations were dissolved in 2% ethanol in distilled water. An equal number of control mice received only 2% ethanol in distilled water. Volumes of drinking water consumed after 24 h were measured. The refusal response (Rr) was defined by the following equation:

$$\text{Rr} = 100 - \left(\frac{\text{avg vol consumed by test group}}{\text{avg vol consumed by control group}} \times 100 \right)$$

where avg vol is average volume. The mice exhibited an Rr to residues obtained from the acetone extracts of rice or corn cultured with *E. chevalieri* or *E. amstelodami*. Only a slight Rr, as determined by the mouse bioassay, was obtained with *A. candidus*. An Rr of <9% [100 − (7.1/7.8 × 100)] was observed for the latter, as compared with an Rr of >35% [100 − (4.9/7.6 × 100)] observed for the *E. chevalieri* and *E. amstelodami* extracts. Rice was selected as the substrate for mass cultures to study refusal factor production because the amount of acetone-extractable substance obtained from the rice culture was not as high as that obtained from the corn culture, as determined by thin-layer chromatography (TLC).

The isolates of *E. chevalieri* and *E. amstelodami* were separately cultured on rice as described above. Extraction with acetone afforded yellow residues weighing 700 and 800 μg/g of rice fermented with *E. amstelodami* and *E. chevalieri*, respectively. Each residue was dissolved in a minimum amount of 95% ethanol. A white solid (corresponding to 33 μg/g of rice fermented with *E. amstelodami*) crystallized at room temperature with a melting point of 241 to 244°C (melting point uncorrected). Similarly, about 30 μg of the same product per g was obtained with *E. chevalieri*. The solid had an R_f of 0.32 in a TLC solvent system containing

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ethyl acetate and hexane (8:2). The spot turned blue in the presence of *p*-anisaldehyde reagent at 110°C.

When offered to mice, the white solid in drinking water at 90 ppm produced a significant ($P < 0.001$) Rr of 36% [100 − (4.9/7.7 × 100)]. The white solid was identified as echinulin by its UV spectrum (λ_{max}^{EtOH}, 231, 279, and 286 nm [ε_{max}, 39,000, 9,600, and 8,990, respectively]), its infrared spectrum (by reflectance infrared spectroscopy on KRS-5 plates), and the absence of a decrease in the melting point of 241 to 244°C when mixed with an authenic sample of echinulin (supplied by R. J. Cole). Its mass spectrum (M^+, 461 atomic mass units (amu); major fragment, 334 amu) was identical to that reported in the literature for echinulin (4).

The feed refused by the swine was examined for echinulin by extraction with acetone (20 g of feed per 100 ml of acetone). TLC analysis of the acetone extract revealed a spot with the same color and R_f after being sprayed with *p*-anisaldehyde as echinulin. A sample of the acetone extract equivalent to 10 g of feed was streaked onto a 2-mm-thick preparative TLC plate (a precoated TLC plate of Silica Gel 60 F-254 with a fluorescent indicator; E. Merck AG, Darmstadt, Federal Republic of Germany). The band corresponding to echinulin was cut from the plate and extracted with acetone. The anisaldehyde-reactive material from the preparative TLC plate was identified as echinulin by its UV spectrum (λ_{max}^{EtOH}, 231, 279, and 286 nm) and by its infrared spectrum. An estimated echinulin concentration of 8 μg/g of feed was determined based on the UV spectrum extinction coefficient at a λ_{max}^{EtOH} of 231 nm. Duplicate feed samples were analyzed and compared with uncontaminated feed samples (containing no detectable amounts of echinulin) amended with echinulin at 4, 8, 12, and 20 μg/g. Recoveries of echinulin in the amended feed samples were 88, 82, 78, and 70%, respectively, when determined in the same manner as that described for the feed sample.

Aspergillus species are often common and prevalent in the mycoflora found in stored feed products because of their ability to grow at moisture contents between 13.5 and 18.5% (3, 8). Of particular interest is the *A. glaucus* group, because of its ability to initiate growth at minimum moisture levels and produce metabolic water, thus paving the way for less xerophytic molds such as *A. candidus* and some *Penicillium* species (8). Some members of the *A. glaucus* group, for example, *A. chevalieri* and *A. amstelodami*, are considered to be mycotoxigenic (4). The principal metabolite of these *Aspergillus* spp. is echinulin, an indole alkaloid (11).

Strains of the *A. glaucus* group grown on cereal grains in various laboratories have been demonstrated to be toxic for experimental animals: *A. amstelodami*-fermented corn was lethal for rabbits (7); *A. amstelodami*-fermented wheat was lethal for mice and, when present in dietary soybeans, *A. amstelodami* led to lowered weight gains in chickens (10); and an extract of *A. amstelodami*-fermented wheat was toxic for chicken embryos (12). *A. chevalieri* grown on various grains also caused dermatoxicity (wheat [2]), death of calves (bread [2]), hemorrhaging in chickens (wheat [9]), toxicity for 1-day-old ducklings (corn [5]), and heptatoxicity in mice (rice [5]). This is the first report of the alkaloid echinulin in swine feed. Since the toxic effects of this alkaloid are unknown, further studies are important, since this alkaloids may adversely affect feed consumption by swine.

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