The presence of mycotoxins in silage is a concern because it may promote the development of infectious diseases in livestock. Silage is frequently contaminated with fungi of the genera Monascus, Aspergillus, and Penicillium (14). One of the most common molds is Penicillium roqueforti, which can produce secondary metabolites such as roquefortine C, isotoxamiclavines A and B, PR toxin, macrofornites, and mycophenolic acid (5, 6, 10, 13). Roquefortine C has been detected frequently in silage (3, 11, 16), but little is known about the natural occurrence of the other mycotoxins, especially mycophenolic acid.

Mycophenolic acid [6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl)-4-methyl-4-hexenoic acid] is a weak organic acid with antifungal, antibacterial, and antiviral activities (1, 2, 3639). Mycophenolic acid is also a noncompetitive inhibitor of eukaryotic inosine monophosphate dehydrogenase (12) and blocks the conversion of inosine-5-phosphate and xanthine-5-phosphate to guanosine-5-phosphate. As an inhibitor of purine rather than on the purine salvage pathway, mycophenolate blocks their proliferative response and inhibits both antibody formation and the production of cytotoxic T cells (9).

Consumption of immunosuppressive compounds increases the risk of infectious diseases in livestock, but this risk cannot be accurately estimated without knowledge of naturally occurring immunosuppressants such as mycophenolic acid in silage. Therefore, we analyzed samples of grass and maize silage for the presence of P. roqueforti and mycophenolic acid.

**Samples.** Samples of grass (n = 98) and maize (n = 135) silage partly visibly contaminated with molds were collected in Bavaria during 1997 and 1998. The mycobiota of the samples was determined quantitatively and qualitatively, and an aliquot of each silage type (500 g) was stored at 18°C until the subsequent dilutions (1:10) were made in sterile peptone water. For mold count determinations, 0.1-ml aliquots from the dilutions (10⁻² to 10⁻⁴) were plated on Sabouraud 2% dextrose agar (Merck) supplemented with 400,000 IU of penicillin G (Sigma, Deisenhofen, Germany) and 40 mg of streptomycin (Sigma) per liter and on DG18 agar (antibiotic-free dichloran-18% glycerol agar base; Oxoid, Wesel, Germany) supplemented with 200 g of glycerol (Merck) per liter and 20 mg of chlorotetracycline (Sigma) per liter. The plates were incubated at 20°C for at least 14 days. Dominant fungal genera and species were identified by macroscopic and microscopic criteria (7, 15).

**Extraction procedures.** A 50-g portion of a well-mixed sample was placed in a 500-ml Erlenmeyer flask with 250 ml of chloroform. The flask was stoppered with a screw cap and shaken on a wrist action shaker for 60 min before filtering of the sample through fluted filter paper (595 1/2; Schleicher & Schuell, Dassel, Germany). An aliquot of 20 ml (equivalent to 4 g of silage) was transferred to a 100-ml round-bottom flask and evaporated to near dryness by rotary evaporation at 35°C.

**Cleanup chromatography.** Mycophenolic acid was purchased from Sigma and used without further purification. All of the solvents used for extraction, cleanup, and liquid chromatography-mass spectrometry (LC-MS) were analytical grade. High-performance liquid chromatography quality water was prepared using a Millipore Milli-Q purification system (Millipore, Eschborn, Germany). Silica gel 60 and sodium sulfate (Na₂SO₄) were obtained from Merck.

**Cleanup column preparation.** Five milliliters of n-hexane was added to a glass column (10 mm [inside diameter] by 300 mm [length] with 35-μm-pore-size porous polypropylene frit and a nylon stopcock), and 0.5 g of anhydrous Na₂SO₄ was added. One gram of silica gel 60 was slurried with 10 ml of hexane in a 15-ml beaker and poured into the column. The beaker was washed twice with 5 ml of solvent to effect transfer. After the gel settled, it was topped with 1 g of anhydrous Na₂SO₄ and the solvent was drained to the top of the Na₂SO₄ layer.

**Cleanup chromatography.** The extract was dissolved in 1 ml of chloroform and transferred to the silica column. The column was washed with 10 ml of hexane–10 ml of toluene–10 ml of toluene-acetone (9.48:0.52, vol/vol); mycophenolic acid was eluted with 40 ml of toluene-acetone-98% acetic acid (30:8:2, vol/vol/vol) into a 100-ml round-bottom flask. The eluate was evaporated to dryness by rotary evaporation at 35°C, and the residue was redissolved in 1 ml of methanol.
LC-MS. The LC-MS system used consisted of a high-performance liquid chromatography pump (2248; Pharmacia LKB, Uppsala, Sweden), a Nucleosil C18 column (125 by 3 mm [inside diameter], 3 μm [Macherey-Nagel, Düren, Germany], ambient temperature), and a quadrupole mass spectrometer (VG Platform 2; Fisons Instruments, Manchester, United Kingdom) equipped with an electrospray ionization source and a MassLynx data system (Fisons Instruments). The mobile phase consisted of acetonitrile-water-100% formic acid (99:99:2, vol/vol/vol). The flow rate was 0.5 ml/min, so postcolumn splitting was arranged to achieve a flow of 20 μl/min to the source. A 10-μl volume of a sample or a standard solution was injected onto the column, and the eluent was monitored either in full scan mode (m/z 100 to 400) or by selected-ion recording. Identification of mycophenolic acid in spiked and native samples was based on the retention time and relative peak area of four selected ions (m/z 321 [M+H]+, 303, 275, 207); in addition, the m/z 343 (M+Na)+ and 359 (M+K)+ ions were monitored. For quantification, the area of the base peak (m/z 303) was compared to that of an external standard.

Validation of analysis. Samples of grass and maize silage were spiked with mycophenolic acid to obtain concentrations of 5 to 500 μg/kg; unspiked silage samples were used as controls. The samples were analyzed, and the recovery rates were calculated.

Examination of the mold flora of 233 silage samples showed that 88% of the samples contained more than 10^5 CFU/g; in most (64%) of the samples, more than 10^6 CFU/g were recovered (Fig. 1). A variety of molds were isolated, especially species of the families dematiaceous Hyphomycetes (n = 34) and Mucoraceae (n = 57), as well as representatives of the genera Aspergillus (n = 35), Penicillium (n = 123), Monascus (n = 43), and Scopulariopsis (n = 16). P. roqueforti was the dominant mold; 70 (30%) samples were contaminated with this species; Aspergillus fumigatus (9% of samples positive) and Monascus ruber (19% of samples positive) were isolated less frequently.

The LC-MS method employed for mycophenolic acid analysis was reliable and linear in a range of 25 to 500 μg/kg. The detection limit was 20 μg/kg (signal-to-noise ratio, 5:1). The selected ion chromatograms of unspiked silage had background components from the matrix, but at the retention time of mycophenolic acid (5.1 min), no interfering ions (m/z 207, 275, 303, 321) were detected (Fig. 2). The mean recovery of
Mycophenolic acid was 85% in maize silage and 86% in grass silage.

Mycophenolic acid was present in 74 (32%) of 233 samples at levels ranging from 20 to 35,000 mg/kg (Table 1). Simultaneous detection of mycophenolic acid and *P. roqueforti* was possible in only 32 samples, and there was no correlation between the fungal counts of *P. roqueforti* and the concentration of mycophenolic acid (data not shown). These results can be explained by the fact that different subsamples were used for mycological investigations and mycotoxin analysis. Moreover, not all strains of *P. roqueforti* produce mycophenolic acid (4) and positive strains produce different amounts under standardized conditions (8). In addition, the CFU counts obtained by dilution plating are related only to the presence of viable conidia and not necessarily to the ability to produce mycophenolic acid. Our results demonstrate that mycophenolic acid occurs frequently in silage. Considering that cattle eat up to 25 kg of silage per day, a dose of 1.8 mg of mycophenolic acid per kg of body weight results. This amount is equivalent to 10% of the dose used to suppress graft rejection in humans. Further study is required to determine if the levels found in silage are high enough to induce immunosuppression in farm animals, resulting in a higher incidence of infectious diseases.

### REFERENCES


### TABLE 1. Occurrence of mycophenolic acid in maize and grass silage

<table>
<thead>
<tr>
<th>Type of silage</th>
<th>No. of samples analyzed</th>
<th>Mean (range) concn (µg/kg) of mycophenolic acid</th>
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<td>Total</td>
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