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Levels of Bacteria, Fungi, and Endotoxin in Bulk and Aerosolized Corn Silage

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Three samples of silage taken from the surface of a silo and from depths of 20 and 45 cm in the silo were studied for identification of the potential agents causing symptoms of organic dust toxic syndrome. The samples were examined by dilution plating before and after aerosolization in an acoustical dust generator. Aerosol samples were collected by liquid impinger and filter cassettes. The samples were examined for total aerobic bacteria, anaerobic bacteria, gram-negative bacteria, lactobacilli, listeriae, thermophilic actinomycetes, fungi, and endotoxin. Very high levels of total aerobic bacteria and fungi were found in the surface sample (up to 10^9 CFU/g in the bulk sample and up to 10^9 CFU/m^3 after aerosolization), whereas the corresponding values from the deepest site were 100 to 50,000 times lower. *Aspergillus fumigatus* predominated among the fungi, whereas *Bacillus* and gram-negative organisms (*Pseudomonas, Alcaligenes, Citrobacter*, and *Klebsiella* species) prevailed among bacteria. Thermophilic actinomycetes occurred in numbers up to 10^7 CFU/g in the bulk samples, whereas anaerobic bacteria, lactobacilli, and listeriae were only few or absent. The concentration of endotoxin was high in the surface sample (up to 211.4 Endotoxin Units/mg) and about 200-fold lower in the sample from the deepest site. The results show that contact with dust from the surface of silage carries the risk of exposure to high concentrations of microorganisms, of which *A. fumigatus* and endotoxin-producing bacteria are the most probable disease agents.

The acute respiratory disease originally described as silo unloader's syndrome (26) and later classified as organic dust toxic syndrome (6) is related to the inhalation of high concentrations of silage dust contaminated with microorganisms and their products (6, 11, 18). Although the clinical disorder has been well described, relatively little is known about the composition of the microflora of silage and potential disease agents.

During silo unloading, the levels of microorganisms in the air are in the range of 10^5 to 10^9 CFU/m^3 (18; J. J. May, D. S. Pratt, L. Stallones, P. R. Morey, S. A. Olenchock, I. W. Deep, and G. A. Bennett, *in* J. A. Dosman and D. W. Cockroft (ed.), *Health and Safety in Agriculture*, in press). Silage-borne molds and mycotoxins were early candidates as causative agents of the respiratory disease in exposed farmers (11). Later studies, however, revealed only trace amounts of such mycotoxins as deoxynivalenol (18; May et al., in press), ochratoxin, and aflatoxin (10) in silage, as well as a lack of correlation between the presence of a mycotoxin and the occurrence of respiratory symptoms (18).

Emanuel et al. (10) expressed the opinion that proteolytic enzymes produced by the thermophilic actinomycete *Thermoactinomyces candidus* might exert a pathogenic action on the people exposed to dust from silage. Olenchock et al. (23, 24) found that the air inside silos and the dust from silage contained high levels of bacterial endotoxin, far exceeding the safe level.

The microflora of silage has been studied to a limited extent by argicultural and veterinary microbiologists (4, 12–14, 19). Coliform bacteria, bacilli, clostridia, lactobacilli, and streptococci were found in various numbers (14, 19) during different stages of the ensilage process. Yeasts and molds were identified as the main organisms causing aerobic deterioration in the regions of silage which are exposed to air (19). Cole et al. (4) reported that the moldy corn silage which had evoked an acute toxic syndrome in beef cattle contained numerous strains of *Aspergillus fumigatus* which produced biologically active mycotoxins: fumigaclavine and tremorgens belonging to the fumitremorgen group. The pathogenic zoonotic bacterium *Listeria monocytogenes* was frequently isolated from silage and is believed to cause a nervous disorder in ruminants. Fenlon (12, 13) found that this bacterium may occur in silage in numbers exceeding 12×10^3/g, being most numerous in the moldy sites of aerobic deterioration.

The purpose of this study was to determine the concentration and species composition of the microflora found in bulk and aerosolized corn silage. The study included screening both for microorganisms which are usually recovered from the samples of plant dusts (aerobic mesophilic bacteria, thermophilic actinomycetes, and fungi) and for those which are only occasionally considered in the studies of organic dusts (anaerobic bacteria, lactobacilli, and *Listeria*-like bacteria) but whose presence might be expected in silage (19). Broad microbiological analysis of silage and silage dust could be helpful in determining potential respiratory biohazards, as was the case with hay dust (15, 17, 25, 34), cotton dust (29, 30), and grain dust (5, 7, 8, 17).

MATERIALS AND METHODS

Silage samples. Three samples of corn silage were taken from a silo located near Cooperstown, N.Y. This was a concrete stave silo, 4.9 by 15.2 m, filled without the use of a plastic cap. The silage had been stored in it for 6 months

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before the sampling was done. The top surface of the silage pile looked moldy, and clouds of white dust were stirred up when it was moved. Respiratory protection was used in this silo to reduce the risk of organic dust toxic syndrome.

The samples for this study (S-1, S-2, and S-3) were taken from the surface of the silage pile (S-1), from a depth of ca. 20 cm below the surface (S-2), and from a depth of ca. 45 cm below the surface (S-3). Each sample was divided into two subsamples, which were placed in separate jugs. One of them, labeled aerobic, was examined for aerobic and facultatively anaerobic bacteria and fungi, while the other one, labeled anaerobic, was examined for anaerobic bacteria. The jugs containing the anaerobic subsamples were flooded with gaseous nitrogen immediately after sampling and sealed for transport to the National Institute for Occupational Safety and Health laboratory. The pH of the silage was determined by using a digital ionalyzer (model 501; Orion Research, Inc., Cambridge, Mass.), with samples suspended in pH 7.00 buffer (Orion). The pH values for samples S-1, S-2, and S-3 were 7.00, 7.13, and 6.70, respectively.

Examination of bulk silage. The concentration of bacteria and fungi in silage was determined by dilution plating. A 1-g portion of each sample (except those for anaerobic bacteria) was suspended in 100 ml of sterile phosphate-buffered saline (Sigma Chemical Co., St. Louis, Mo.) amended with 0.1% Tween 80 (Fisher Scientific Co., Fair Lawn, N.J.), and after vigorous shaking, serial 10-fold dilutions were made up to 10^{-8}. Then, 0.1-ml portions of each dilution were spread on duplicate sets of the following agar media: (i) and (ii), two sets of blood agar made by supplementing of blood agar base no. 2 (Difco Laboratories, Detroit, Mich.) with 5% defibrinated sheep blood, set (i) for total aerobic mesophilic bacteria and set (ii) for total facultatively anaerobic mesophilic bacteria; (iii), CDC anaerobe blood agar for anaerobic bacteria (before inoculation, the anaerobic subsample was suspended and diluted in reduced transport fluid [33], and the medium was inoculated in a glove box with an anaerobic atmosphere); (iv), eosin methylene blue agar (EMB agar; Difco), and (v), Hektoen enteric agar (Difco), both for gram-negative bacteria; (vi), Rogosa SL agar (Difco), and (vii), tomato juice agar (Difco), both for lactobacilli; (viii), half-strength tryptic soy agar (Difco) for thermophilic actinomycetes; (ix) and (x), two sets of rose bengal streptomycin agar (RBS) (28), set (ix) for total mesophilic fungi and set (x) for total thermophilic and thermotolerant fungi; (xi) and (xii), two sets of Czapek solution agar (Difco) with the same purpose as above; and (xiii) and (xiv), two sets of malt agar with the same purpose as above.

Most media for bacteria [(i), (iv), (v), (vi), and (vii)] were incubated under aerobic conditions for 48 h at 35°C after inoculation. Set (ii) for facultatively anaerobic bacteria was incubated in a 10% CO_2 atmosphere for 72 h at 35°C. Set (iii) was incubated in the glove box under an anaerobic atmosphere (CO_2, NO_2, H_2) for 72 h at 35°C. Set (viii) was incubated for 120 h at 55°C. The sets of media for mesophilic fungi [(ix), (xi), and (xiii)] were incubated for 120 h at 28°C, and the sets for thermophilic and thermotolerant fungi [(x), (xii), and (xiv)] were incubated for 120 h at 45°C.

In addition to the above procedures, a procedure described by Fenlon (12, 13) was used for isolation of *Listeria* species. Portions (1 ml) of each sample were added to enrichment broth containing nalidixic acid, potassium thiocyanate, and acriflavine (all from Sigma). After preincubation of the inoculum for 48 h at 35°C, 25-μl portions were taken from the cultures showing visible growth, plated on selective blood agar with nalidixic acid and acriflavine, and incubated for 48 h at 35°C.

Following incubation, bacterial colonies were counted and differentiated on the basis of colony morphology and Gram reaction. Gram-positive isolates were identified to the generic or specific level by microscopic, biochemical, and serological methods selected from *Bergey's Manual* (31). Gram-negative isolates were identified to the specific level by using API Systems 20E (for enterobacteria) and NFT (for nonfermenting bacteria) (Analytab Products, Plainview, N.Y.), with supplementary biochemical tests selected from *Bergey's Manual* (16) and API Systems recommendations. The fungal colonies were counted and differentiated on the basis of morphological features. Final results for microbial concentrations were reported in CFU per gram of silage.

For endotoxin determination, 1-g portions were taken from each aerobic and anaerobic subsample of silage. The portions were extracted separately with 100 ml of sterile nonpyrogenic water (Travenol Laboratories, Deerfield, Ill.) by rocking for 60 min at room temperature. The suspension was centrifuged at 1,000 × *g* for 10 min to remove particulate debris, and the supernatant fluid was separated for further analysis. The gram-negative bacterial endotoxin content was quantitated in duplicate by using a chromogenic modification of the *Limulus* amebocyte lysate gel test (QCL-1000; Whittaker M.A. Bioproducts, Walkersville, Md.), and the results were reported in terms of endotoxin units (EU) per milligram of silage.

Examination of aerosolized silage. The system described by Sorenson et al. (32) was used for aerosol generation and analysis. Respirable-sized dust particles were generated from 1-g portions of silage samples S-1, S-2, and S-3 in a miniature version of the Pitt-3 (35) acoustical dust generator. This minigenerator has a length of Plexiglas tube (10 by 3.5 in. [inner diameter] [ca. 25 by 8.9 cm]), with vibration provided by rubber dam material over a 2.5-in. (ca. 6.3-cm) speaker, powered at 115 Hz and 3 V. The dust was carried from the generator by an airflow of 1 liter/min.

An aerodynamic particle sizer (model 3300; TSI, Inc., St. Paul, Minn.) was used to measure size distribution (mass and number) of dust particles throughout the run. The sizer was interfaced with an Apple II^+ computer with all data stored on floppy disks for future analysis. A 5-s sample time at an airflow of 5 liters/min was used.

Preweighed Metricel 37-mm polyvinyl chloride filters (Gelman Sciences, Inc., Ann Arbor, Mich.) were used to collect dust for gravimetric analysis and the endotoxin assay. The dust was pulled through the filters with a portable pump (model P2500; Du Pont Co., Wilmington, Del.) at a flow rate of 1.0 liter/min. After dust collection on the filters, they were weighed again, and the change in weight and gravimetric concentration of the respirable dust (reported in terms of milligrams per cubic meter of outgoing air) was reported. Afterwards, they were used for the endotoxin assay.

For estimation of the concentration of bacteria and fungi in aerosolized silage, the respirable-sized dust particles were collected into two serially connected midget impinger washers filled with sterile phosphate-buffered saline amended with 0.002% Tween 80 as the absorbent liquid, with an airflow of 1.0 liter/min. After sampling, the contents of both washers were pooled and mixed. Serial 10-fold dilutions were made, and 0.1-ml portions were plated in duplicate on the following agar media, prepared as described above: blood agar for aerobic mesophilic bacteria, EMB agar for gram-negative bacteria, Rogosa SL agar for lactobacilli,

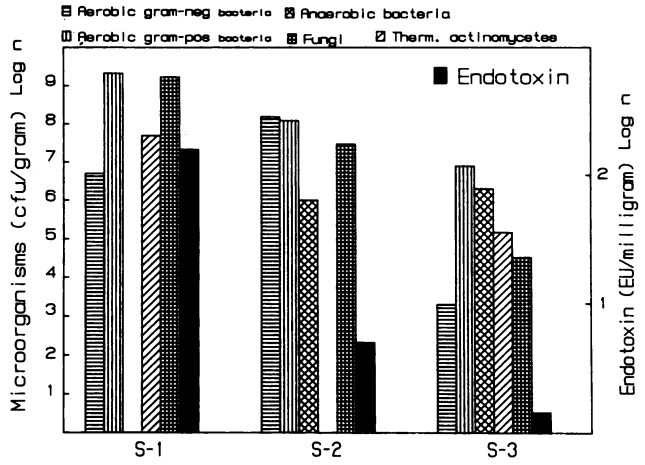

FIG. 1. Concentrations of gram-negative and gram-positive bacteria (blood agar), thermophilic actinomycetes (tryptic soy agar), fungi (RBS agar, mesophilic), and endotoxin (determined by the *Limulus* chromogenic method, mean values) in bulk silage samples S-1, S-2, and S-3, shown as logarithmic values.

selective agar for *Listeria* species (using different inoculation doses as described above), half-strength tryptic soy agar for thermophilic actinomycetes, and RBS agar for mesophilic fungi. The methods for incubation and examination of the inoculated media and for identification of the isolates were the same as those given above. Based on colony count and on gravimetrically determined dust concentration in the outcoming air, the results were reported both in terms of the CFU per cubic meter of dust-contaminated air sampled from the generator and in terms of the CFU per gram of respirable dust.

For determination of the endotoxin content in respirable dust, the dust-bearing filters were extracted separately with 10 ml of sterile, nonpyrogenic water by rocking for 60 min at room temperature, and the above-described determination procedure was then carried out.

RESULTS

Concentration of microorganisms and endotoxin in bulk silage. The concentrations of microorganisms and endotoxin in bulk silage are shown in Fig. 1 and Tables 1 to 4. It may be seen that the total concentrations of aerobic bacteria, fungi, and endotoxin were very high in the surface (uppermost) layer of the silage pile and then rapidly dropped in deeper layers, being on average 100- to 50,000-fold lower in the deepest layer (45 cm below the suface) (Fig. 1; Tables 1 and 4). *Bacillus* species predominated among bacteria isolated from the uppermost layer (Table 1). Gram-negative bacteria were much less numerous there, with *Alcaligenes faecalis* being the most common species (Table 2).

Unexpectedly, the number of gram-negative bacteria was more than 10-fold greater in the subtopical layer (S-2) than in the surface layer (S-1). Gram-negative organisms constituted the prevalent fraction of the total bacterial flora isolated from sample S-2 (Table 1), the most common species being *Pseudomonas putida* and *Citrobacter diversus* (Table 2). The gram-negative organisms disappeared almost completely over the short distance between 20 and 45 cm below the surface of the silage pile. Their concentration dropped by 50,000-fold to a level of 10^3 CFU/g in sample S-3, with *Achromobacter* being the only genus identified.

TABLE 1. Composition of aerobic bacteria in bulk silage[a]

Aerobic bacteria	No. (10^6 CFU/g) (%) in sample:		
	S-1	S-2	S-3
Gram-negative bacteria	5.0 (0.2%)	155.1 (55.7%)	0.002 (0.03%)
Staphylococcus spp.	0	1.6 (0.6%)	0
Bacillus spp.	2,125.0 (99.8%)	30.0 (10.8%)	7.90 (99.97%)
Coryneform bacteria (*Arthrobacter* spp., *Corynebacterium* spp., and others)	0	90.0 (32.3%)	0
Streptomyces	0	1.3 (0.4%)	0
Other bacteria	0	0.6 (0.2%)	0

[a] The bacteria were incubated on blood agar. Counts for facultatively anaerobic mesophilic bacteria (incubation in 10% CO_2) did not show major differences from the results reported above (data not shown).

The level of anaerobic bacteria in the silage samples studied was very low, but, compared with the rest of the microorganisms, it tended to increase with the depth of the pile and was highest at the deepest site examined (Table 3). No lactobacilli were found in any of the silage samples.

The low level of *Listeria*-like bacteria growing on the selective medium was found in sample S-2 from the subtopical layer. These bacteria agglutinated the polyvalent anti-*L. monocytogenes* serum (Difco). However, the identification tests (made by R. E. Weaver, Centers for Disease Control, Atlanta, Ga.) did not confirm their identity with *L. monocytogenes*, and the taxonomic position of these bacteria remains unclear.

Thermoactinomyces vulgaris was the only thermophilic actinomycete found in the silage. Its concentration was high in the surface layer and then dropped rapidly.

Of the fungi, the sole dominant species was *A. fumigatus*, which occurred in sample S-1 in large concentrations, exceeding 10^9 CFU/g (Table 4). The concentration of this

TABLE 2. Composition of gram-negative bacteria in bulk silage[a]

Gram-negative bacteria	No. (10^6 CFU/g) (%) in sample:		
	S-1	S-2	S-3
Enterobacteria			
Citrobacter diversus	0	40.0 (36.4%)	0
Enterobacter amnigenus	0	5.0 (4.5%)	0
Klebsiella oxytoca	0	5.0 (4.5%)	0
Providencia rettgeri	1.2 (16.4%)	0	0
Other gram-negative bacteria			
Achromobacter xylosoxidans	0	1.2 (1.1%)	0.002 (100%)
Alcaligenes faecalis	3.6 (49.3%)	1.2 (1.1%)	0
Pseudomonas maltophilia	0.5 (6.9%)	0	0
Pseudomonas putida	0	45.0 (41.0%)	0
Pseudomonas stutzeri	0	10.0 (9.1%)	0
Other *Pseudomonas* spp.	2.0 (27.4%)	0.5 (0.4%)	0
Unidentified	0	2.1 (1.9%)	0

[a] The bacteria were incubated on EMB agar. Counts on Hektoen enteric agar were 30 to 60% lower than reported above and did not show the presence of any additional species (data not shown).

TABLE 3. Concentration of certain fastidious bacteria in bulk silage

Fastidious bacteria (growth medium)	No. (10^6 CFU/g) in sample:		
	S-1	S-2	S-3
Anaerobic bacteria (CDC anaerobe blood agar)[a]	0	1.0	2.0
Lactobacilli (Rogosa agar, tomato juice agar)	0	0	0
Listeria-like gram-positive rods (Fenlon isolation procedure)	0	≥0.01	0
Thermophilic actinomycetes (*T. vulgaris*) (half-strength tryptic soy agar)	50.0	0	0.15

[a] Colonies of aerobic bacteria (mostly *Bacillus* spp.) which are not recognized in this table were found on most plates.

fungus then fell to 10^7 CFU/g in the subtopical layer (S-2) and to 10^4 CFU/g in the deepest layer (S-3).

The concentration of endotoxin was highest in sample S-1 (104.07 EU/mg in the aerobic subsample and 211.43 EU/mg in the anaerobic subsample) and then fell rapidly, being, on the average, 30 times lower in sample S-2 (6.79 and 3.12 EU/mg, respectively) and 100 times lower in sample S-3 (1.09 and 1.79 EU/mg, respectively). This pattern followed the concentration of total bacteria more closely than that of gram-negative bacteria, which was highest in sample S-2.

Concentration of the microorganisms and endotoxin in aerosolized silage. The aerosolization of samples S-1 and S-2 of silage released large amounts of respirable dust, resulting in concentrations of 552.5 and 825.0 mg/m^3, respectively. The corresponding concentration of respirable dust from sample S-3 was only 5.3 mg/m^3. The median particle size (aerodynamic diameter) estimates for samples S-1, S-2, and S-3 were 2.1, 1.2, and 3.4 μm, respectively. The aerosol particles generated from sample S-1 were very small, being less than 2.5 μm for both diameter at peak number concentration and mass median aerodynamic diameter (Fig. 2). Particles in this size range are able to penetrate deep into the human lungs, increasing the potential respiratory risk associated with inhalation of this dust.

The concentrations of the particular microorganisms and endotoxin in the air polluted with the respirable dust from silage (expressed in terms of CFU per cubic meter and EU per milligram) are shown in Fig. 3, whereas the concentration and composition of the microflora in the respirable dust itself (expressed in terms of millions of CFU per gram) are presented in Tables 5 to 7. It may be seen that experimental aerosolization of the silage sample from the surface layer (S-1) released into the air vast number of microorganisms,

TABLE 4. Concentration of fungi in bulk silage[a]

Fungi (incubation temp)	No. (10^6 CFU/g) in sample:		
	S-1[b]	S-2[c]	S-3[d]
Mesophilic fungi (28°C)	1,700.0	23.0	0.034
Thermophilic or thermotolerant fungi (45°C)	1,700.0	30.0	0.078

[a] The fungi were incubated on RBS agar. Counts on Czapek solution agar and malt agar did not show major differences from the results reported above (data not shown).

[b] Fungal flora consisted almost exclusively of *A. fumigatus*.

[c] Fungal flora consisted almost entirely of *A. fumigatus*, with only a few *Acremonium* colonies.

[d] Fungal flora was more diverse, but *A. fumigatus* was still the predominant species (the remaining fungi were not identified).

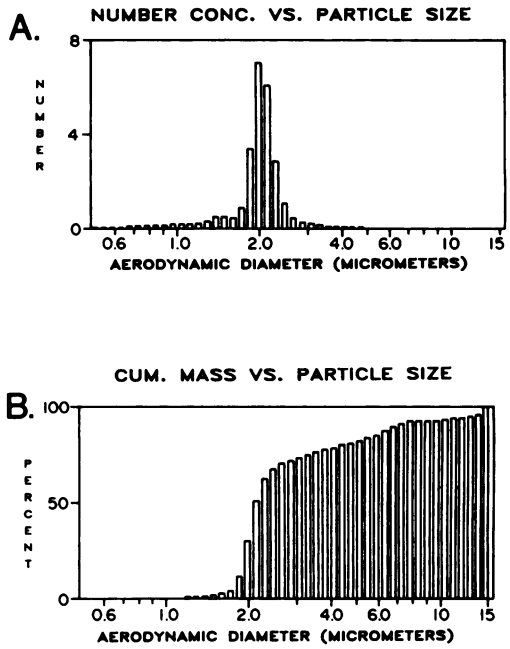

FIG. 2. Computer-generated plots of number distribution (A) and mass distribution (B) of aerosol particles from the silage sample S-1. Both the diameter at peak number concentration and the mass median aerodynamic diameter are less than 2.5 μm.

exceeding 10^9 CFU/m^3 of the air. Aerosolization of the silage samples from the subtopical and deepest layers resulted in numbers of microorganisms about 300- and 3,000-fold lower, respectively.

The concentration of bacteria per unit weight of respirable dust was lower in most cases than in bulk silage (Table 5), except for gram-negative bacteria in sample S-1, which were more than 33-fold more numerous in the respirable dust (Table 6). The concentration of gram-negative bacteria in that dust reached the high level of 2.44×10^8 CFU/g, whereas in the air polluted with this dust the level was 1.35

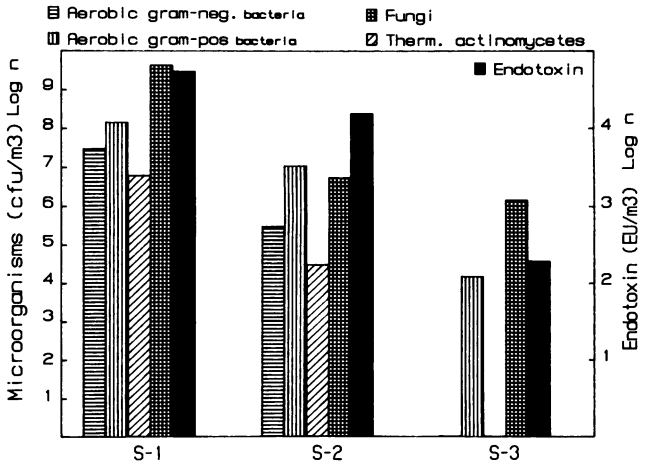

FIG. 3. Concentrations of gram-negative and gram-positive bacteria (blood agar), thermophilic actinomycetes (tryptic soy agar), fungi (RBS agar), and endotoxin (determined by the *Limulus* chromogenic method) in the air polluted with the respirable dust generated from silage, shown as logarithmic values.

TABLE 5. Concentration and composition of aerobic bacteria in the aerosolized respirable dust from silage[a]

Aerobic bacteria	No. (10^6 CFU/g) (%) in sample:		
	S-1	S-2	S-3
Gram-negative bacteria	54.2 (17.1%)	0.4 (2.9%)	0
Staphylococcus spp.	54.2 (17.1%)	0.2 (1.5%)	0.47 (16.6%)
Bacillus spp.	208.3 (65.8%)	12.4 (90.5%)	2.36 (83.4%)
Streptomyces spp.	0	0.7 (5.1%)	0

[a] The bacteria were incubated on blood agar.

$\times 10^8$ CFU/m^3 (Table 6; Fig. 3). By contrast, only a small proportion of the gram-negative bacteria present in bulk silage sample S-2 could be detected in the respirable dust.

The species composition of the gram-negative bacteria in the respirable dust was different from that in bulk silage. The dominant species was *Pseudomonas maltophilia* (Table 6), which might indicate that this species could be released into the air much more easily than *Pseudomonas putida* and members of the family *Enterobacteriaceae*, which were common in the samples of bulk silage.

Except in sample S-3, *A. fumigatus* constituted the predominant part of the airborne microflora released from silage, far above 90% of the total (Table 7). The concentration of *A. fumigatus* spores per unit weight of the respirable dust released from the most contaminated sample (S-1) (7.69×10^9 CFU/g) was more than fourfold higher than in bulk silage (Table 7). The concentration of *A. fumigatus* in the air polluted with this dust reached the very high level of 4.25×10^9 CFU/m^3.

The thermophilic actinomycetes were about fourfold less common in the respirable dust than in bulk silage. Their concentration in the air polluted with this dust was 6.15×10^6 CFU/m^3. The concentration of the *Listeria*-like bacteria was about 12-fold higher in the respirable dust than in bulk silage; nevertheless, their level in the air did not exceed 1.0×10^5 CFU/m^3.

The descending levels of endotoxin concentration in the air polluted with the respirable dust from silage samples S-1 to S-3 were similar to those of gram-negative bacteria (Fig. 3), being highest in sample S-1 and lowest in sample S-3. However, the differences between the samples in endotoxin concentration per unit weight proved to be smaller in the respirable dust than in bulk silage. The values of the endotoxin concentration in the respirable dust from samples S-1, S-2, and S-3 were 98.49, 18.85, and 36.19 EU/mg, respectively.

TABLE 6. Concentration and composition of particular bacteria growing on special media in the aerosolized respirable dust from silage

Bacteria (growth medium)	No. (10^6 CFU/g) (%) in sample[a]:		
	S-1	S-2	S-3
Gram-negative bacteria (EMB agar)			
Pseudomonas maltophilia	195.48 (80.0%)	0.036 (24.8%)	0
Other *Pseudomonas* spp.	29.81 (12.2%)	0.042 (29.0%)	0
Alcaligenes faecalis	19.06 (7.8%)	0.012 (8.3%)	0
Unidentified	0	0.55 (37.9%)	0
Listeria-like gram-positive rods (Fenlon isolation procedure)	0	0.121	0
Thermophilic actinomycetes (*T. vulgaris*) (half-strength tryptic soy agar)	11.13	0.036	0

[a] Percentages are given in terms of the gram-negative bacteria only.

TABLE 7. Concentration and composition of mesophilic fungi in the aerosolized respirable dust from silage[a]

Fungi	No. (10^6 CFU/g) (%) in sample:		
	S-1	S-2	S-3
A. fumigatus	7,690.0 (100%)	6.31 (96.3%)	7.2 (2.6%)
Monosporium sp.	0	0.24 (3.7%)	0
Penicillium sp.	0	0	261.1 (94.8%)
Mucor sp.	0	0	7.2 (2.6%)

[a] The fungi were incubated on RBS agar at 28°C.

DISCUSSION

The bulk silage we examined contained large concentrations of potentially pathogenic microorganisms and endotoxin. The concentration of microorganisms was highest in the surface layer (over 10^9 CFU/g), being comparable to microbial levels reported from highly contaminated plant materials (hay, grain, and clover) that caused symptoms of hypersensitivity pneumonitis and/or organic dust toxic syndrome in exposed agricultural workers (15, 20, 34). This finding also conforms with the earlier observations that the most severe symptoms of organic dust toxic syndrome appear when the farmers are uncapping a silo to prepare for unloading and are inhaling the dust released from the surface layer of silage (18, 24, 26, 27; May et al., in press). The microorganism content in silage fell rapidly with the depth of the pile, and at only 45 cm below the surface it was about 1,000-fold lower than at the surface. These data suggest that unloading of a silo becomes safer after the top layer, which is highly contaminated down to 20 to 30 cm, is removed.

The potential risk of disease is enhanced by the composition of the microflora of silage, which included large numbers of molds and bacteria reported as hazardous agents (4, 17, 25, 30). *A. fumigatus* constituted up to 43% of the organisms living in the uppermost layer. This species may occur abundantly in different organic dusts (3) and has been reported as a cause of pulmonary mycosis (21) and of allergic respiratory diseases (17) in people exposed to these dusts. It is also known to produce mycotoxins (4, 36).

The silage also contained considerable numbers of microorganisms belonging to two other groups known to be dust-borne respiratory hazards: gram-negative bacteria of plant origin having allergenic and endotoxic properties (5, 8, 29), and thermophilic actinomycetes of the species *T. vulgaris*, which is one of the best-known agents of hypersensitivity pneumonitis (17, 25). Among the gram-negative bacteria found in the silage, some species or genera have been reported as a putative source of endotoxin in different materials or dusts: *P. putida* in flax (30), *Alcaligenes faecalis* in herbs (9), *Klebsiella* spp. in compost (3) and cotton (30), and *Citrobacter* spp. in dust from an animal farm (1). The composition of the gram-negative microflora that we report for corn silage was different from that reported by Gibson et al. (14) for grass silage, which was dominated by *Klebsiella* spp., *Escherichia coli*, and *Enterobacter agglomerans*. It is interesting that *Enterobacter agglomerans* (syn. *Erwinia herbicola*), which is reported as the main source of endotoxin in the dusts from cotton (29, 30) and grain (5, 9), was not found in our samples of corn silage. The importance of the gram-negative bacteria in the silage we examined was enhanced by the finding of high levels of biologically active

endotoxin by the *Limulus* test. The concentration of endotoxin in the uppermost layer proved to be very high, many times higher than in the subtopical layer, which had a higher proportion of gram-negative bacteria. This apparent discrepancy most probably could be explained by the exposure of the surface layer to desiccation, which might kill the vulnerable living cells of gram-negative bacteria, while the thermoresistant endotoxin persisted.

The possible role of other bacteria present in the silage, including the abundant *Bacillus* sp., as a respiratory biohazard is less clear. The anaerobic bacteria and lactobacilli were rare or absent and most probably do not represent a risk, at least in silage with a pH close to neutral. The significance of corynebacteria and *Listeria*-like bacteria, which were found in one of the samples, is unclear but merits further consideration. It was recently found that coryneform bacteria of the genus *Arthrobacter* could cause respiratory allergy (20).

The aerosolization experiments confirmed the high degree of potential respiratory risk connected with handling the upper layer of silage. Aerosolization of the sample taken from this layer generated large amounts of fine dust containing many more potentially inflammatory fungi and gram-negative bacteria per unit weight than the bulk silage did. Air polluted with this dust contained 4.25×10^9 spores of *A. fumigatus* per m^3, 1.35×10^8 gram-negative bacteria per m^3, and 54.4×10^3 EU/m^3. These values exceeded the suggested safe levels for fungi, gram-negative bacteria, and endotoxin (2) by about 40-, 100,000-, and 50-fold, respectively (assuming that 1 ng of endotoxin equals approximately 10 EU). The experimentally estimated concentrations of microorganisms and endotoxin in the airborne dust from silage conforms to the levels found by Olenchock et al. (23, 24) and May et al. (in press) in field studies performed during unloading of silos.

The specific roles of the predominating exposures of agricultural workers to endotoxins and *A. fumigatus*, including potential synergistic interactions of both agents, during unloading of silos must be defined further. It is also possible that endotoxins may act in this environment as adjuvants, enhancing immunostimulative properties of other as yet undefined antigens. Thus, the physiologic responses in the workers could be due to the nonspecific activation of pulmonary macrophages, cellular components of the immune system, and complement system by endotoxins and fungal antigens (22).

Our results confirm the substantial microbiological risk posed by allowing surface silage to grow mold and dry out. The use of appropriate capping of a silo (for example, with plastic sheets) may reduce the degree of this risk. The data also strongly support the suggestion by May et al. (in press) for farm workers to use National Institute for Occupational Safety and Health/Mine Safety and Health Administration-approved dust and mist respiratory protective equipment while unloading silos.

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