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Dust-borne bacteria in animal sheds, schools and children's day care centres

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A.M. ANDERSSON, N. WEISS, F. RAINEY AND M.S. SALKINOJA-SALONEN. 1999. A total of 316 bacterial strains, including psychrophiles, mesophiles and thermophiles, were isolated and identified from indoor dusts in schools, children's day care centres and animal sheds. Several species which had not previously been reported from indoor environments were found: *Sphingomonas, Brevibacterium*, *Nocardiopsis*, *Deinococcus* and *Rhodococcus/Gordona*. A new psychrophilic actinomycete genus was also found in animal sheds, representing a new undescribed peptidoglycan type and an unusual whole-cell fatty acid composition. The indoor dusts of animal sheds contained mainly the Gram-negative genera *Pseudomonas, Pantoea, Flavobacterium* and *Xanthomonas* early in the indoor feeding season, but changed to a composition dominated by *Bacillus, Micrococcus* and mesophilic and thermophilic actinomycetes towards the end of the season. The dust contained, and air-borne bacterial flora in schools and day care centres were dominated by, Gram-positive bacilli and actinomycetes, notably *Bacillus cereus, Brevibacillus brevis, B. licheniformis, B. subtilis* and species of *Arthrobacter, Corynebacterium, Rhodococcus/Gordona, Nocardiopsis* sp., *Deinococcus, Staphylococcus* and *Micrococcus*. Indoor air and dust contained *Klebsiella oxytoca*, *Acinetobacter calcoaceticus, Ac. lwoffi, Bacillus cereus* and *Nocardiopsis dassonvillei* with the status of hazard group II. Indoor dusts of animal sheds contained eight different 3-hydroxy fatty acids, the 2-hydroxy fatty acid 14:0 and two 10-methyl fatty acids, whereas in dusts from schools and day care centres, these were below the detection level ($< 3{\cdot}5$ ng mg^{-1}). The 3- and 2-hydroxy fatty acids could be assigned to one or more of the dust-contained cultivable strains, but 10-methyl C16:0 was not present in any of the strains isolated. The dusts from schools and children's day care centres contained 0·2–0·3 ng of endotoxin mg^{-1} and 0·5–1·4 ng of β-D-glucan mg^{-1}, whereas the dusts from animal sheds contained more 0·3–41 ng mg^{-1} and 8–35 ng mg^{-1}, respectively.

INTRODUCTION

Association of occupational diseases with microbially-contaminated indoor air from animal sheds, and school and children's day care centre buildings with a history of moisture damage, has long been suspected by health authorities (Donham 1994; Kauppinen *et al.* 1994; Lacey 1994; Rylander 1996; Taskinen *et al.* 1997). Water-damaged indoor building materials are frequently colonized by complex bacterial communities and may emit mixed bioaerosols into the indoor air (Lacey 1994; Andersson *et al.* 1997).

The assessment of bacterial aerosols has mainly focused on the total amount of air-borne bacteria, or on a few hazardous agents such as endotoxin or β-glucan (Olenchock *et al.* 1990; Donham 1994; Dutkiewicz *et al.* 1992; Rylander 1996). In most studies, air-borne microbes have been cultivated with no attempt to maximize the yield of cultivable bacteria by resuscitation. The cultivable part of bioaerosols described in these studies may represent a highly selected population of species resistant to environmental stress. Only 0·1–10% of the bacteria counted by direct microscopic techniques may

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be cultivable (Palmgren *et al.* 1986; Saraf *et al.* 1997). Choice of the method used for sampling bioaerosols will affect both the number of viable organisms and the species composition obtained by cultivation (Marthi 1994; Stewart *et al.* 1995) because air-borne and dehydrated bacteria are sublethally injured. In addition, the majority of studies do not apply modern methods of classification. Therefore, the present knowledge of bacterial species diversity in aerosols is fragmentary and may be heavily biased.

Dust-borne bacterial diversity in environments with high and low air-borne concentrations of organic dust, namely animal sheds, schools and children's day care centres, were studied. The dusts were resuscitated to maximize culturability and several temperatures were used for cultivation. A total of 316 bacterial strains were identified to species or genus level, and several genera not previously reported from indoor aerosols are described. A good match was found between the culturable bacteria, the dust-contained signature fatty acids and the amounts of *Limulus*-reactive, Gram-negative endotoxin.

MATERIALS AND METHODS

The cattle barns ($n = 4$) were representative of dairy farming in southern Finland, housing about 10–20 cows and lacking mechanical ventilation. The swine confinement buildings ($n = 2$) were large units (mechanically ventilated) housing about 300 free-living animals in an area of 50 m × 100 m covered by a thick layer (> 1 m) of wood chips. The schools and day care centres ($n = 9$), housing 50–300 children each, had a history of water damage and/or building related occupational health problems, and were located in southern Finland. The study included old and new buildings, with and without mechanical ventilation.

Sampling

Animal sheds. Dusts aerosolized during the distribution and handling of feed and bedding were collected onto nuclepore filters as described by Palmgren *et al.* (1986). Baled hay (harvested in June–July) was used as feed and baled straw (harvested in September–October) was used as bedding. Only good quality (not visibly mouldy) feed and bedding materials were used. The storage temperature of the bales ranged from + 20 – 20 °C during the season. The humidities of the bales were approximately 60%. The cow barn aerosols were collected for 10 min on nuclepore filters, with a nominal pore size of 0·2 μm, using a low flow personal sampling pump precalibrated to a flow of 2 l min^{-1} as described by Andersson *et al.* (1995). Sampling was done at a distance of 0·4 m from the bales of hay and straw being opened; the air contained visible clouds of dust. Air-borne dust was similarly collected in swine confinement buildings during the weekly turning over of the bedding of wood chips by a bulldozer, which generated a visible cloud of dust in the air. The humidity of the bed of wood chips was 70–95%, and the sampling time was 2 min at a distance of 1–20 m from the working bulldozer. Settled dusts (approximately 200 mg) were collected from horizontal surfaces in the animal sheds, i.e. the feeding table, and fences at 1 m and > 2 m above the floor.

Public buildings. Air-borne dusts were collected according to Andersson *et al.* (1995) during the ordinary working day. Settled dusts (approximately 100 mg) were collected from surfaces located 1·5–2·5 m above the floor level.

Electron microscopy

For scanning electron microscopy (SEM), about 100 mg settled hay or straw dust were equilibrated in 10 ml resuscitation medium (Andersson *et al.* 1995), drained on nuclepore filters (0·2 μm) and fixed in 2·5% glutaraldehyde in potassium phosphate buffer (0·1 mol l^{-1} pH 7·3, Merck) for 1 h. The filters were washed with the same buffer, dehydrated in a graded series of ethanol (50–99%) for 2 min each of the five steps, and for 10 min in 100%, and incubated in hexamethyldisilazane (Fluka 52620) in a Petri dish with the lid open for 24 h. Samples of hay were fixed for 8 h in vapour phase 20% glutaraldehyde. The samples were critical-point dried, coated with gold for 6 min and examined in a Jeol 180 scanning electron microscope (Tokyo, Japan) at an operating voltage of 5–7 kV. Particles of the size and shape of bacterial cells were enumerated from five randomly selected electron micrographs from each filter.

Media and chemicals

The microbiological media and diluent for serial plating are as those described in Andersson *et al.* (1995) unless otherwise stated. The reference endotoxin *Escherichia coli* B4 lipopolysaccharide was from Sigma. Other chemicals were from local suppliers and were of analytical quality.

Microbiological analyses

Bacteria in settled and air-borne dusts were resuscitated by a method described previously (Andersson *et al.* 1995) and cultured on tryptic soy agar at 16, 22 and 50 °C. The resuscitation method yielded viable counts of 40–80% of the microscopic counts (Laukkanen *et al.* 1987). Isolated mesophilic bacterial strains were Gram-stained according to Shab *et al.* (1984); they were analysed for endotoxin by the *Limulus* reaction and for whole cell fatty acids. *Limulus*-positive, negatively Gram-staining strains which were shown by FAME to contain 3-hydroxy fatty acids were recorded as endotoxin-

containing Gram negatives, and *Limulus*-inactive strains lacking 2- or 3-hydroxy fatty acids were recorded as Gram positives. Negatively Gram-staining, *Limulus*-inactive strains containing 2-hydroxy fatty acids were recorded as endotoxin-lacking Gram negatives. The strains were identified to genus or species level by methods described elsewhere (Greiner-May *et al*. 1987; Andersson *et al*. 1995,1997; Smibert and Krieg 1994). Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA, and purification of PCR products, were carried out as described by Rainey *et al*. (1992). Purified PCR products were sequenced as described in Andersson *et al*. (1997) and Andersson *et al*. (1998b). The EMBL accession number of the almost complete sequence was strain 801=Y 18807, and for the partial sequences strain 8/PP1=Y 17513, strain 10/PP1=Y 17514 and strain 703=Y 17517.

Gram-negative endotoxin and β-D-glucan were simultaneously estimated by *Limulus* reactivity from dusts, as described by Andersson *et al*. (1997) using E-Tect endotoxin kits (Farmos, Turku, Finland), and calibrated with *E. coli* B4 LPS. The *Limulus* activity of 1 ng *E. coli* B4 was equivalent to that of 34 000 cfu *E. coli* O157–4430/88 cells (range 10 000–59 000). The strain O157–4430/88 was isolated from bovine mastitis and obtained from A.J.S. Van Miert (State University of Utrecht, The Netherlands). Upon repeated testing, the limit of detection of the E-Tect test averaged 0·1 ng of LPS ml^{-1} (range 0·04–0·2 ng ml^{-1}).

Analysis of the cell wall (peptidoglycan structure) was carried out according to the methods described by Schleifer (1985), with the modification that thin-layer chromatography on cellulose was used instead of paper chromatography. Amino acids and peptides from the partial and total hydrolysates were identified after two-dimensional chromatography, as described by Schleifer (1985), by their mobilities and staining characteristics with ninhydrine spray.

Fatty acid composition

Whole-cell fatty acid composition and 2- and 3-hydroxy fatty acids in settled dust were quantified as methyl esters by gas liquid chromatography by the same procedure as that used for pure cultures (Andersson *et al*. 1995). The assay was calibrated by adding 32 and 80 mg *E. coli* B4 LPS and 24, 60 and 120 μg heptadecanoic acid as internal standards to parallel samples of dust (200 mg) before methylation and extraction. The detection limit for 3-hydroxy fatty acids was 3·5 ng mg^{-1} dust. All test tubes and reagents were pretested to exclude contamination.

RESULTS

Air-borne and settled dusts were collected from urban and agricultural indoor environments and analysed, after resuscitation, for aerobic heterotrophic culturable bacteria, endotoxin and β-D-glucan. The strains obtained were identified at genus and/or species level.

Contents of viable aerobic dust-borne bacteria in animal sheds, schools and children's day care centres

Figure 1 shows the densities of cultivable air-borne and dust-contained aerobic bacteria from five animal sheds and nine schools and day care centres. Settled dusts (Fig. 1a) from animal sheds contained 10^3–10^7 cfu mg^{-1} of cultivable aerobic mesophilic heterotrophic bacteria, which is a 10^2–10^4-fold higher density than that found in the dust from schools and day care centres. The content of cultivable, endotoxin-containing, Gram-negative bacteria in the dusts of the animal sheds was 10^2–10^5 times higher (depending on the season) than in the dusts from schools and day care centres (Fig. 1a). The dusts from animal sheds were also richer in facultatively and obligately thermophilic bacteria.

Figure 1(b) shows the air-borne densities of cultivable, aerobic, heterotrophic bacteria from the same buildings. It can be seen that the animal shed air contained 10^5–10^9 cfu of cultivable heterotrophic bacteria m^{-3}, while the air in the urban public buildings contained 10^2–10^3 cfu m^{-3}. The difference was even higher for the Gram negatives. Thermophiles were not found in the air from schools and day care centres.

The results show that endotoxin-containing, Gram-negative bacteria were frequent in animal sheds, both airborne and in settled dusts. The air-borne and the dust-contained, cultivable bacteria in schools and day care centres consisted almost exclusively of Gram-positive bacteria. The numbers of cultivable, facultatively and obligately thermophilic bacteria did not correlate with those of the total cultivable bacteria in air or in settled dusts in either type of buildings.

Aerobic bacterial taxon diversity in the air and in the settled dusts of the animal sheds, schools and children's day care centres

Of the 316 strains isolated from dust and air of the animal sheds, schools and day care centres, 80% were assigned to a genus or a species using whole-cell fatty acid composition, phenotypic characterization, *Limulus* reactivity, 16S rDNA sequencing (eight strains) and composition of cell-wall peptidoglycan (four strains). The taxa and their prevalence in the indoor samples are listed in Table 1.

Table 1 shows that *Bacillus, Arthrobacter, Micrococcus, Acinetobacter, Deinococcus, Nocardiopsis*, *Rhodococcus* and related actinomycetes were found both air-borne and in settled dusts, indicating high stress survival of these bacteria in the dehydrated and aerosolized state. *Pseudomonas* and *Pantoea* appeared to be characteristic for animal sheds. These genera

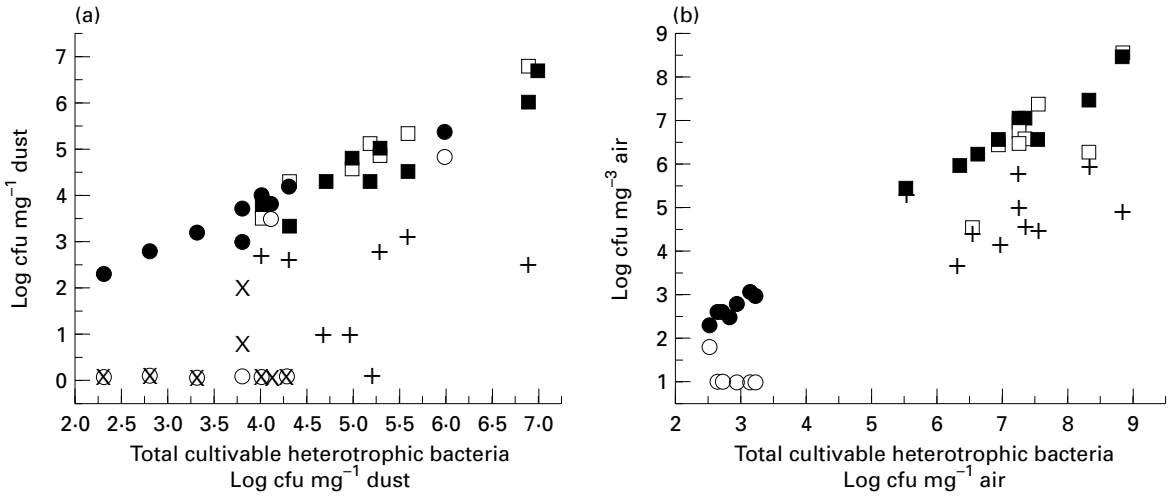

Fig. 1 Cultivable bacteria from animal sheds and urban public buildings (schools and children's day care centres). The figure shows the contributions of Gram-positive, endotoxin-containing, Gram-negative and facultatively and obligately thermophilic cultivable bacteria (in animal sheds and urban buildings, respectively) to the total heterotrophic viable count in (a) indoor dust and (b) air. The cultivable Gram-negative and Gram-positive bacteria were counted after 7–14 d of incubation at 22 °C, and the thermophilic, after 7 d at 50 °C. The Gram-negative bacteria (endotoxin-containing) were distinguished from the Gram-positive by *Limulus* reactivity and presence of LPS specific 3-hydroxy fatty acids in whole cells. (□), Gram negatives (animal sheds); (■), Gram positives (animal sheds); (+), thermotrophs (animal sheds); (○), Gram negatives (urban buildings); (●), Gram positives (urban buildings); (×), thermotrophs (urban buildings)

and the genus *Cytophaga* were found in settled dusts rather than airborne, indicating low survival in the aerosolized state.

Air-borne and settled dusts in cattle barns contained the same bacterial genera, indicating that the sources of emission were the same for both. Representatives of the endotoxin-containing, Gram-negative genera *Pantoea* and *Pseudomonas* were found in highest densities in dusts of hay and straw cultivated at 22 and 10–16 °C. The dominant Gram-positive genera were *Micrococcus, Arthrobacter*, spore-forming actinomycetes and representatives of a new psychrophilic genus, X, related to *Microbacterium/Aureobacterium* by complete sequencing of 16S rDNA. The fact that new genus X, *Bacillus, Streptomyces, Nocardiopsis dassonvillei, Microbispora* and *Sphingomonas* were more frequently air-borne than other isolated strains may be indicative of better survival in the aerosolized state than the endotoxin-containing Gram negatives. *Bacillus cereus* and *Moraxella osloensis* were isolated from settled dusts in swine confinement buildings but were not air-borne.

The dust-contained and air-borne bacterial flora in schools and day care centres was dominated by Gram-positive bacilli and actinomycetes, notably *B. cereus, Brevibacillus brevis, B. licheniformis, B. subtilis* and species of *Arthrobacter, Corynebacterium, Rhodococcus/Gordona, Nocardiopsis* sp., *Deinococcus, Staphylococcus* and *Micrococcus.* The endotoxin-containing Gram-negative bacteria found in schools and day care centres were *Moraxella osloensis, Klebsiella oxytoca, Enterobacter*, *Acinetobacter calcoaceticus* and *Ac. lwoffii.*

Characterization of the psychrotrophic and psychrophilic dust-borne genera

The viable counts in air and dust from animal sheds, schools and day care centres obtained by cultivation at 10–16 °C and 22 °C were similar and 100–1000-fold higher than those obtained at 50 °C. Many of the strains isolated at temperatures below 16 °C were not easily cultivable at 28 °C and were not recognized by the MIDI-MIS fatty acid commercial library (version 3·9; MIDI Inc., Newark, DE, USA). These strains ($n = 9$), characterized chemotaxonomically and by 16 S rDNA ($n = 8$) sequencing, were identified as strains of *Deinococcus, Cytophaga*, *Sphingomonas,* members of other α-Proteobacteria and actinomycetes. *Sphingomonas* strains ($n = 2$) were identified on the basis of partial 16S rDNA sequencing, absence of *Limulus* reactivity, Gram-negative staining, positive aminopeptidase reaction, and the signature fatty acid C14: 0 2-OH in the whole-cell fatty acid composition. Another α-Proteobacteria, identified as such by the 16S rDNA sequence, positive *Limulus* reactivity and positive aminopeptidase activity, was not assignable to any genus on the basis of this information. *Cytophaga* sp. (two strains) was

Table 1 Genera and species of aerobic heterotrophic bacteria isolated (on tryptic soy agar at 16 °C and 22 °C) after resuscitation from indoor air and settled dusts from animal sheds, schools and day care centres

	Approximate density in indoor air cfu m^{-3}			Approximate density in settled dust cfu g^{-1}		
	Cow shed	Piggery	Schools and day care centres	Cow shed	Piggery	Schools and day care centres
Mesophilic, psychrotropic and psychrophilic Actinomycetes						
Agromyces sp.				10^7		
Arthrobacter ramosus			$10–10^2$		$10^7–10^9$	
Arthrobacter pascens						10^5
Arthrobacter sp.	10^7		10	10^8		10^4
Brevibacterium sp.		10^6	10^2		10^6	
Clavibacter sp.	10^6		10^2	10^5		
Corynebacterium sp.				10^8		10^6
Curtobacterium sp.		10^5			10^5	
Microbispora sp.	$10^7–10^8$					
Nocardiopsis dassonvillei	$10^5–10^7$			10^7		
Nocardiopsis sp.						$10^5–10^6$
New genus X	$10^5–10^8$			10^7		
Rhodococcus/ Gordona		10^5	10^2		10^9	$10^2–10^4$
Rhodococcus fasclans			10^2			$10^2–10^4$
Spirillospora sp.			10^2			
Streptomyces sp.	$10^4–10^6$	10^4	10^2	$10^5–10^8$	$10^6–10^7$	10^6
Mesophilic and psychrotrophic Gram positives						
Bacillus alvei	10^6					
B. cereus					10^5	10^5
B. licheniformis	10^7		10^2	10^6		10^3
B. mycoides	10^6					
B. psychrophilus			10^3			
B. subtilis	10^7	10^6				
Bacillus sp.						10^6
B. thuringiensis	10^6					
Brevibacillus brevis		10^6	10^2			10^4
Deinococcus sp.			10^2			10^4
Micrococcus sp.	10^7	$10^6–10^7$	$10–10^2$	10^8	$10^6–10^9$	10^6
Paenibacillus pabuli						10^5
Staphylococcus sp.	10^6		$10–10^2$	10^8	10^9	10^5
Mesophilic and psychrotrophic Gram negatives (endotoxin positives*)						
Acinetobacter lwoffi			10^2			
Ac. calcoaceticus			10^2			10^2
Acinetobacter sp.	10^8	10^5				
Agrobacterium sp.	10^8					
Brevundimonas sp.				10^9		
Chryseomonas sp.						$10^2–10^3$
Cytophaga sp.					10^9	
Enterobacter sp.			10^2			
Flavobacterium sp.					10^9	
Klebsiella oxytoca			10^2			
Microbulbifer sp.		10^5				
Moraxella osloensis			10^2		$10^5–10^9$	
Pantoea sp.	$10^4–10^7$	10^6		$10^6–10^8$		10^3
α-*Proteobacterium* sp.				10^7		
Pseudomonas chlororaphis	$10^6–10^8$			$10^6–10^7$		
Ps. cichorii					10^6	
Ps. stutzeri	10^8					

Table 1 *(Continued)*

	Approximate density in indoor air cfu m^{-3}			Approximate density in settled dust cfu g^{-1}		
	Cow shed	Piggery	Schools and day care centres	Cow shed	Piggery	Schools and day care centres
Ps. syringae	10^6–10^8			10^7		
Ps. marginalis					10^6	
Ps. putida		10^6				
Xanthomonas sp.	10^8	10^5				
Psychrotrophic Gram negatives (endotoxin negatives*)						
Sphingomonas sp.	10^4–10^5					
Facultatively and obligately thermophilic bacteria						
Bacillus sp.		10^4–10^6	10^2		10^4–10^6	
B. amyloliquefaciens						10–10^2
B. licheniformis	10^5	10^4–10^5		10^6	10^4–10^6	
B. subtilis	10^4	10^4–10^6		10^5	10^4–10^6	
B. pumilus	10^4			10^5		
Brevibacillus brevis						10^2
Paenibacillus macerans	10^4			10^5		
Saccharomonopora sp.		10^3–10^5			10^3–10^5	
Thermoactinomyces sacchariі	10^4–10^6	10^3–10^5		10^5–10^6	10^3–10^5	
Theam. vulgaris	10^4–10^6			10^5–10^6		10^3
Thermomonospora sp.	10^4			10^5		
Cumulative total	65	39	45	72	47	48

**Limulus* reactive endotoxin.

recognizable by the characteristic, branched 3-hydroxy fatty acids in the whole-cell fatty acid composition, positive *Limulus* reactivity and Gram-negative staining.

Four Gram-positive psychrophilic isolates were identified as members of a new actinomycete genus. The strains grew well on tryptic soy agar plates at temperatures below 4 °C. They were related to *Microbacterium/Aureobacterium* by the complete 16S rDNA sequence, a peptidoglycan structure of a hitherto new undescribed B-type (Hsr) D-glu-D-lys, and a whole-cell fatty acid composition containing a high percentage of C14: 0 2-OH, which is unusual in Gram-positive genera. The full taxonomic description of this genus will be reported elsewhere.

Endotoxin and β-D-glucan in settled dusts from animal sheds and children's day care centres

The contents of Gram-negative endotoxin and β-D-glucan in settled dusts from six animal sheds and two day care centres are shown in Fig. 2. The endotoxin contents were high in all animal shed dusts, from 8–35 ng mg^{-1} dust equivalent to 10^5–10^6 cells of *E. coli* O157–4430/88. The β-D-glucan con-

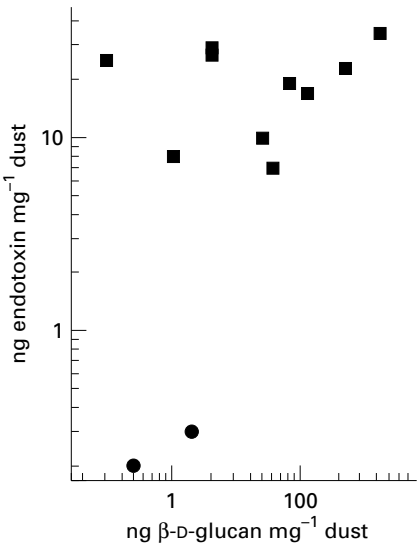

Fig. 2 Contents of endotoxin and β-D-glucan in settled dusts from (■) animal sheds, and (●) schools and children's day care centres (public buildings). The endotoxin and β-D-glucan were simultaneously measured from the same lots of dust as *Limulus* activity using the kinetic turbidometric LAL-assay

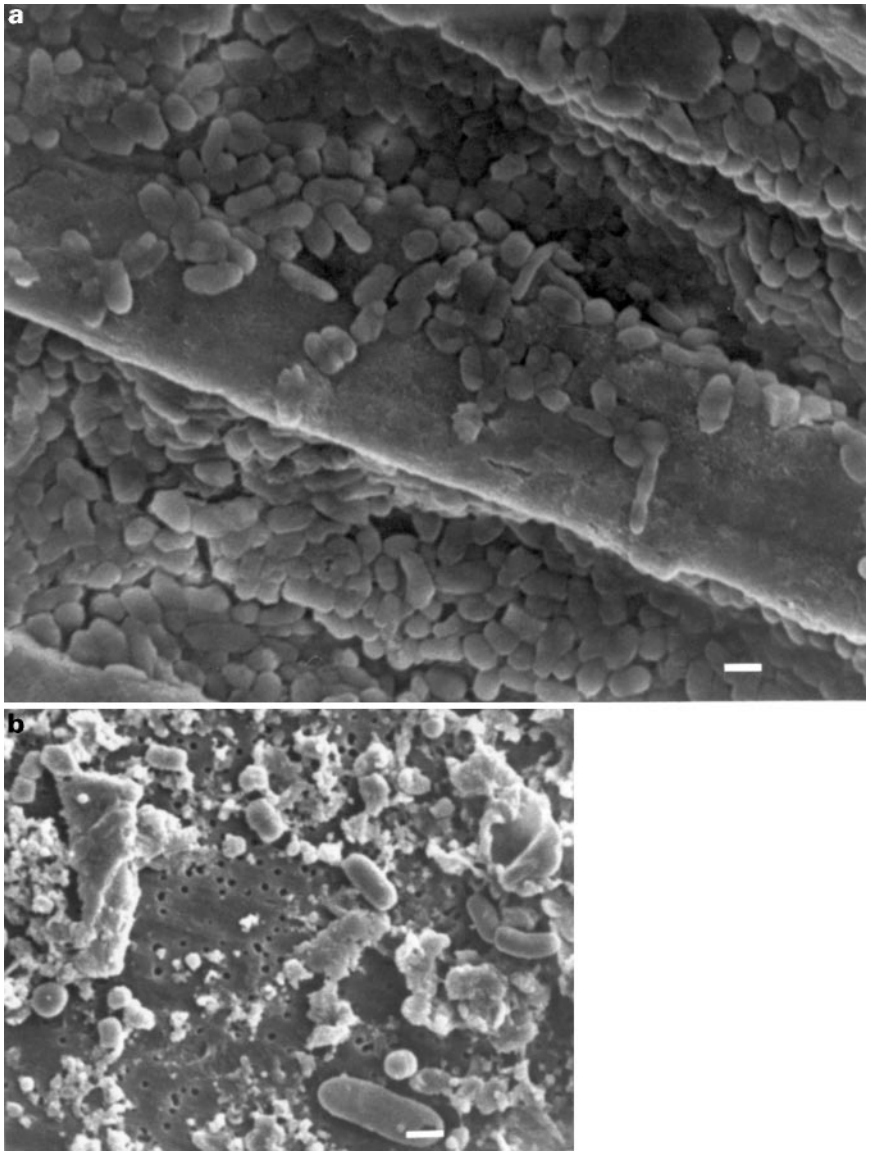

Fig. 3 Scanning electron micrograph of hay feed and hay dust. The top panel shows the surface of good quality dry hay used as feed in the cow barn. The high degree of bacterial colonization is clearly visible. The bottom panel shows dust emitted from hay and straw. The settled dust was rehydrated in a resuscitation solution (+ 4 °C), centrifuged (900 *g*, 5 min) to remove debris, and filtered on a nuclepore filter. The figure shows several rod-shaped and spherical particles resembling bacteria. Bars represent 1 μm

tent ranged from 0·3–2 ng mg^{-1} dust (four dusts) to high, 5–41 ng mg^{-1} (six dusts). There was no correlation between the contents of endotoxin and β-D-glucan contents in the dusts from animal sheds (Fig. 2). Both were low in the dusts from the children's day care centres, i.e. 0·2–0·3 ng of endotoxin mg^{-1} and 0·5–1·4 ng mg^{-1}, respectively.

Figure 3 shows electron micrograph images of the hay (non-mouldy) used as the feed, and of the dust originating from the feed (baled hay) and bedding (baled straw) material used in the cow barn. Hay stems were densely covered by bacteria (Fig. 3, upper panel). The densities of particles in the dust morphologically similar to bacteria were counted from micrographs similar to that shown in Fig. 3 (lower panel), and were found to range from 10^6 to 10^7 mg^{-1} hay and straw dust. The normal healthy hay and straw thus emitted dust heavily loaded with bacteria, which explains the high content of endotoxin prevalent in the cow barns.

Endotoxin indicators in dusts and air from animal sheds, schools and children's day care centres

The endotoxin (LAL-reactivity) and the contents of 3-hydroxy fatty acids were analysed in air and in settled dusts from cow barns, swine confinement buildings, schools and day care centres (Fig. 4). The endotoxin content of the dust ranged from 4 to 2000 ng in the animal sheds, and from 0·16 and 0·006 ng in the urban buildings, expressed as *E. coli* B4 LPS equivalents mg^{-1} dust. Several different 3-hydroxy fatty

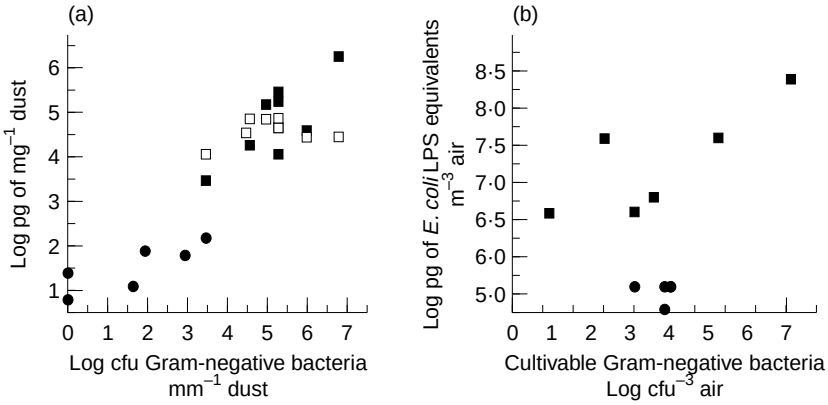

Fig. 4 Contents of endotoxin in settled dusts (a) and air (b) sampled from cow sheds (three), swine confinement buildings (four) and schools and day care centres (urban buildings) (nine). The endotoxin content is shown as ng *E. coli* B LPS equivalents. Contents of endotoxin were assayed as LAL reactivity and 3-hydroxy fatty acids, plotted against the viable count of the endotoxin-containing Gram-negative bacteria. Cultivation of Gram-negative bacteria was as for Fig. 1. (a) Shows that the correlation of LAL reactivity with the cultivable Gram-negative bacteria, was for settled dusts in animal sheds, very significant ($P = 0{\cdot}008$, $r = 0{\cdot}845$) and less significant in urban public buildings ($P = 0{\cdot}034$, $r = 0{\cdot}745$). (■), *Limulus* reactivity in cowsheds; (□), 3-hydroxy fatty acids in animal sheds; (●), *Limulus* activity in urban buildings. (b) Shows that air-borne LAL reactivity in cowsheds correlated with the amount of air-borne viable Gram-negative bacteria, but was not statistically significant ($P = 0{\cdot}063$, $r = 0{\cdot}78$). In swine confinement buildings, there was no correlation. (●), Endotoxin in swine confinement buildings; (■), endotoxin in cowsheds

acids were found in the dusts sampled from the animal sheds (Table 2) but no 3-hydroxy fatty acids (above the detection level of 3·5 μg of 3-hydroxy fatty acids g^{-1} of dust) were found in settled dusts from scools and day care centres. The content of 3-hydroxy fatty acids was invariably high in animal shed dusts, with no correlation with the count of cultivable Gram-negative bacteria (Fig. 4a). The dusts from animal sheds contained 100–1000 times more endotoxin than the dusts from schools and day care centres, and the endotoxin content of these dusts correlated with the count of cultivable Gram-negative bacteria; approximately 0·1 pg *E. coli* B4 LPS equivalent in dusts from schools and day care centres, and 0·25 pg Gram-negative cfu^{-1} in animal shed dusts, were found. Both figures are higher than those measured for freshly cultivated cells of *E. coli* strain O157–4430/88 (0·03 pg LPS cfu^{-1}). This indicates that more than 70% of the endotoxin (LAL reactivity) in the indoor dusts may have originated from sources other than the cultivable portion of the Gram-negative bacteria. The Gram-negative viable count assay thus gives an underestimation of dust-borne endotoxin.

Figure 4(b) shows that contents of air-borne endotoxin ranged from 4 to 2000 μg m^{-3} in the cattle barns, and from 0·2 to 0·4 μg m^{-3} in the swine confinement buildings, during working activities (distribution of feed, turning over of the bedding). The air-borne endotoxin contents in the nine schools and children's day care centres tested were, in all cases, below the detection level of 2 ng m^{-3}, whereas only in two buildings were cultivable Gram-negative bacteria detected (> 30 cfu m^{-3}). Therefore, the contents of air-borne

Table 2 Bacterial signature fatty acids in settled dusts from animal sheds and dust-borne bacterial strains containing the same fatty acids

Signature fatty acid found in dust	Dust-borne bacterial strains
Cow shed	
C10:0 3-OH	*Pseudomonas chlororaphis, Ps. putida, Ps. stutzeri, Ps. syringae, Brevundimonas* sp.
C12:0 3-OH	*Ps. chlororaphis, Ps. putida, Ps. stutzeri, Ps. syringae*
C14:0 3-OH	*Pantoea* sp. *Agrobacterium* sp.
C16:0 3-OH	*Agrobacterium* sp., *Flavobacterium* sp.
C17:0 3-OH	*Flavobacterium* sp.
C12:0 iso 3-OH	*Xanthomonas* sp.
C16:0 iso 3-OH	None isolated
C17:0 iso 3-OH	Spore-forming actinomycete
C14:0 2-OH	*Sphingomonas* sp., New genus X
10 methyl C16:0	None isolated
10 methyl C17:0	*Microbispora* sp.
Swine confinement buildings	
C10:0 3-OH	*Ps. cichorii, Ps. marginalis*
C12:0 3-OH	*Ps. cichorii, Ps. marginalis*
C14:0 3-OH	*Moraxella* sp.
C16:0 3-OH	*Cytophaga* sp.
C17:0 iso 3-OH	*Cytophaga* sp.
C14:0 2-OH	None isolated

endotoxin and Gram-negative bacteria were $\leqslant 10^2$–10^5 times higher in animal sheds than in schools and day care centres.

The air-borne endotoxin content of cow sheds correlated with the amount of air-borne viable endotoxin-containing, Gram-negative bacteria, but were not statistically significant ($P = 0{\cdot}063$, $r = 0{\cdot}78$; Fig. 4b). In swine confinement buildings, there was no correlation. In the air of the cowshed, more than 10 pg *E. coli* B4 LPS equivalents were found per endotoxin-containing, Gram-negative cfu. This is greater than the level found in settled dust ($0{\cdot}25$ pg cfu^{-1}), indicating a higher contribution of non-culturable Gram-negative bacteria.

Seasonal trends in the endotoxin and bacterial content in cattle barn dusts

The endotoxin-containing Gram-negative, Gram-positive and thermophilic culturable bacteria, and endotoxin *Limulus* activity, were quantified throughout the indoor feeding season in a cattle barn. In total, 130 bacterial strains were isolated from the air-borne and settled cattle barn dusts during the whole season.

The results are shown in Figs 5 and 6. The culturable endotoxin-containing, Gram-negative bacteria predominated early in the indoor feeding season and declined as the indoor feeding season advanced, especially in winter, after the hay and straw had been frozen. As the season advanced, the viable counts of the endotoxin-containing Gram-negative bacteria declined more than those of the mesophilic Gram-positives or thermophiles, both in the air and in the settled dusts (Figs 5a and 6a). The viable counts of the thermophilic actinomycetes correlated with the total amounts of thermophiles and represented about 50% of the thermophilic viable count (not shown). The viable counts of the thermophiles were most persistent towards inactivation, both air-borne and in the settled dusts (Fig. 5). A high numbers of non-cultivable mesophilic bacteria may thus be hidden by a high viable count of thermophiles.

Figures 5 and 6 further show that the dust-borne contents of 3-hydroxy fatty acids were stable throughout the season, in contrast to those of the endotoxin (measured as LAL reactivity) which declined to 1/100, and to the Gram-negative viable count, which declined by a factor of 10 000 in the air-borne dust and 100 in the settled dusts. The number of viable, air-borne, endotoxin-containing, Gram-negative bacteria was only 0·1% of that expected on the basis of the air-borne LAL reactivity at the end of the indoor feeding season.

Bacterial diversity in the dusts as reflected by the spectrum of signature fatty acids

Settled dusts from animal sheds contained several 3-hydroxy fatty acids (Table 2), consistent with the presence of Gram-

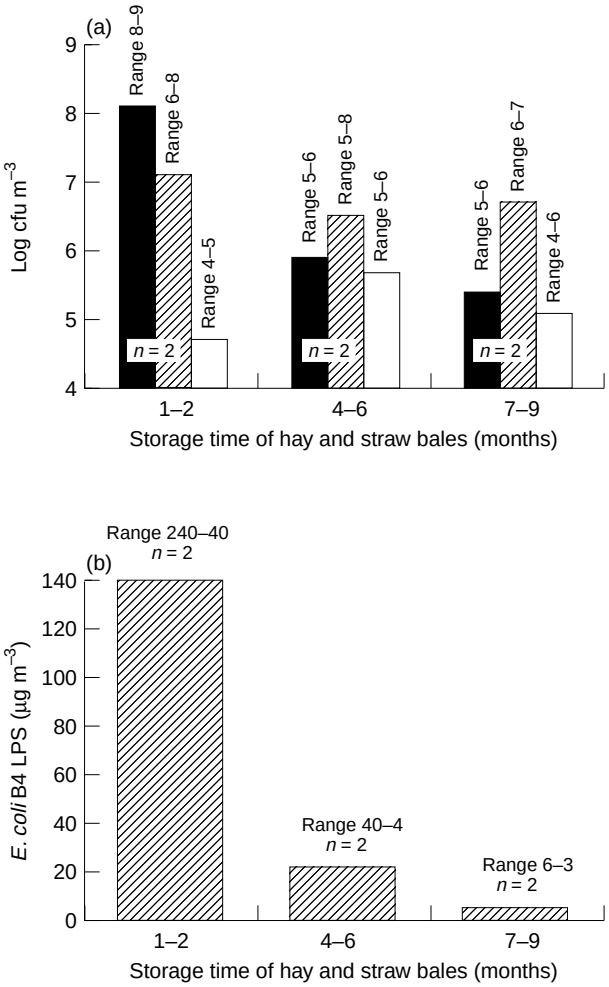

Fig. 5 The air-borne biological activities of dust during the indoor feeding season in a cow barn. The feed and bedding were the main sources of the dust and the season is therefore denoted as the time post-harvest of hay and straw. The cultivation was as for Fig. 1. (a) The viable count of mesophilic (endotoxin-containing) Gram-negative bacteria (■) declined more as the indoor feeding season advanced than that of mesophilic Gram-positive bacteria (▨) and facultative and obligate thermophiles (□), both in air-borne and settled dusts. (b) *Limulus* activity during the indoor feeding season

negative bacteria as indicated by the high dust-borne LAL reactivity and the high Gram-negative viable counts (Fig. 4). Strains of *Cytophaga* in settled dusts from swine confinements (Table 1) contained the fatty acids C16: 0 iso 3-OH and C17: 0 iso 3-OH, respectively. These same fatty acids were found in cow barn dust, indicating the presence of uncultivable *Cytophaga*. The C14: 0 2-OH was regularly found in settled dusts from cow sheds and swine confinement buildings in large quantities (5–20 μg g^{-1}), indicating the presence of the new genus related to *Microbacterium*/*Aureobacterium* or of *Sphingomonas*.

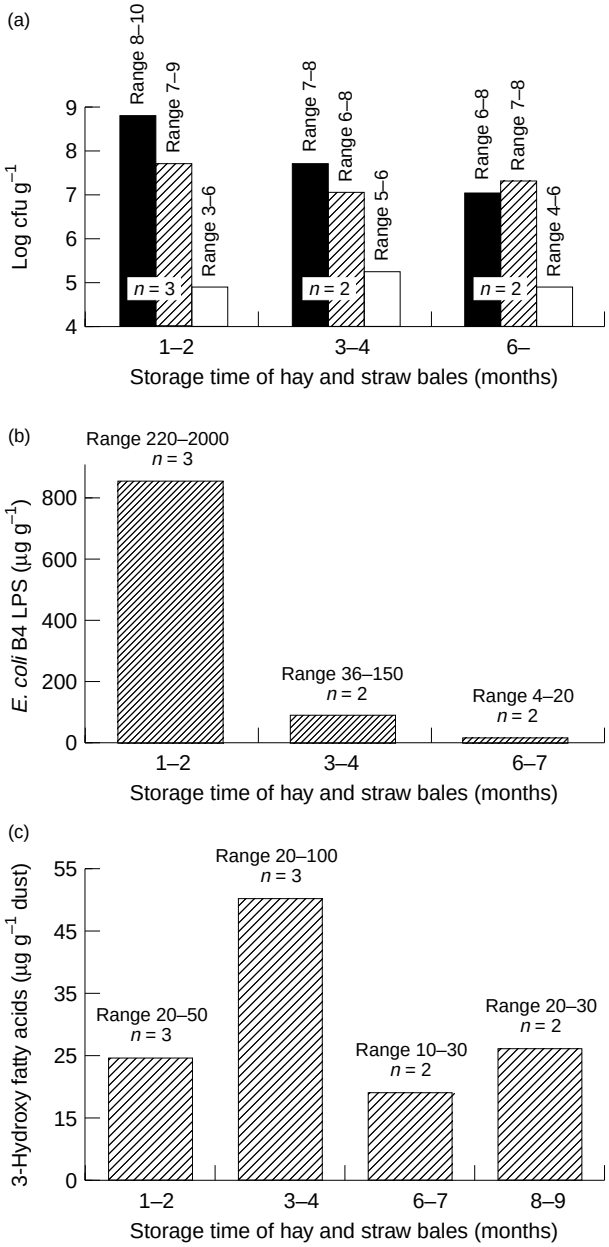

Fig. 6 (a) The compositions and biological activities of settled dusts in a cow barn during the indoor feeding season. The feed and bedding were the main sources of the dust and the season is therefore denoted as the time post-harvest of the hay and the straw. The cultivation was as in Fig. 5. (■), Gram negatives; (▨), Gram positives; (□), thermophiles. Endotoxin content is shown both as LAL reactivity (b) and as the content of 3-hydroxy fatty acids (c)

The results in Table 2 further show that the cow shed dust, but not swine confinement building dust, contained 10 methyl C16: 0, indicative of nocardioform actinomycetes but not found in any of the strains detected by cultivation.

DISCUSSION

Indoor aerosols contained a diverse bacterial flora and many bioactive agents

In this study, the bioaerosols in regularly functioning animal sheds, and in schools and day care centres considered by the local health authority to have a mould problem, were analysed by uniform methods.

To maximize the yield of different air-borne taxa, the dehydrated dust-borne bacteria were resuscitated prior to cultivation and the plates were incubated at different temperatures. Sub-optimal incubation decreased competition and inhibition between neighbouring colonies, and may have increased the detectable number of species (Chang *et al.* 1995). Injured, dehydrated bacteria in bioaerosols may not grow on selective media or at selective pH (Mackey 1984; Marthi 1994). Glucose-free universal media were also used instead of the widely used standard media (Greenberg *et al.* 1992) because reducing sugars have been shown to inhibit dehydrated (membrane-damaged), Gram-positive and Gram-negative bacteria (Mackey 1984; Chmielewski and Frank 1995), and to increase colony masking as described by Chang *et al.* (1995).

Several genera which, to our knowledge, had not previously been reported from indoor environments or water-damaged buildings, species of *Deinococcus*, *Microbulbifer*, *Sphingomonas, Gordona* and *Nocardiopsis*, and a new psychrophilic actinomycete genus, were found. Animal sheds contained mainly endotoxin-containing, Gram-negative bacteria and high amounts of mesophilic and thermophilic actinomycetes. Endotoxin, in combination with the immunopotentiating cell wall components of actinomycetes, has been proposed as the causative agent for farmers' lung (Fogelmark *et al.* 1989; McNeil and Brown 1994). High amounts of *Sphingomonas* were also found airborne in cow sheds. Sphingolipids, present in cell walls of *Sphingomonas* and *Sphingobacterium,* are known to be toxic to eukaryotic cells (Hannun and Bell 1989).

A guideline has been proposed for air-borne, *Limulus*-active endotoxin of 30 ng m^{-3} (Palschack *et al.* 1990), and for viable air-borne bacteria, of 10^3–10^4 cfu m^{-3} (Flannigan *et al.* 1991; Maroni *et al.* 1995), for occupational environments. These levels were grossly exceeded in the animal sheds during the distribution of feed and bedding. The high exposure may explain the observed long-term excess of respiratory diseases by Finnish farmers (Notkola *et al.* 1987; Malmberg 1990; Tammilehto *et al.* 1994).

Schools and day care centres contained mainly bacilli and mesophilic actinomycetes. Several of the Gram-positive taxa shown in Table 1, *Bacillus cereus* (Andersson *et al.* 1998a; Granum and Lund 1997), *B. subtilis (*Crupper *et al.* 1997) and *B. licheniformis* (Yakimov *et al.* 1996; Vuorio *et al.* 1998), and species of *Brevibacillus brevis* (Gräfe 1992), *Staphylococcus* (Vol-

lenbroich *et al.* 1997) and *Streptomyces griseus* (Andersson *et al.* 1998b), are known as potential producers of toxic and bioreactive agents. Nocardioform actinomycetes were found in swine confinement buildings and schools. Cell-wall components of the genera found, *Rhodococcus*, *Corynebacterium* and *Gordona,* as well as *Mycobacterium*, are known to stimulate the immune system (Ridell 1983; Shoenfield and Isenberg 1988; Vuorio *et al.* 1999). These potentially immunoreactive agents may be emitted into indoor air and dust, possibly contributing to building-related illness in indoor environments where the contents of endotoxin and viable microbes are relatively low, as exemplified by schools and day care centres in this study.

Nocardiopsis dassonvillei, Ac. calcoaceticus, Ac. lwoffi, B. cereus and *Kl. oxytoca* have been classified to hazard group II as opportunistic pathogens (Advisory Committee on Dangerous Pathogens 1995). *Nocardiopsis dassonvillei* was found in animal sheds in this study; it has also been isolated from a lung biopsy and is suspected of causing extrinsic alveolitis (McNeil and Brown 1994). Our study revealed the presence of hazard group II bacteria in large quantities (Table 1) in indoor air in schools and day care centres.

Viable, endotoxin-containing, Gram-negative bacteria, *Limulus* activity and 3-hydroxy fatty acids as indicators of endotoxin exposure

The viable count of Gram-negative bacteria decayed faster in bioaerosols than that of Gram-positive bacteria as the indoor feeding season of the cow sheds advanced. Facultatively and obligately thermophilic bacteria gradually became more dominant in the cow barn air. Loss of endotoxin (*Limulus* reactivity) (Douwes *et al.* 1995) and culturability upon freeze-thawing and aerosolization of Gram negatives has also been recognized in other studies (Mackey 1984; Bale *et al.* 1993; Marthi 1994).

Tween-80 in pyrogen-free water has been shown to increase extraction efficiency of endotoxin from filters and dusts compared with extraction by pyrogen-free water only (Douwes *et al.* 1995). In a study in which Tween was not used for extraction, between 0·4 and 4 μg endotoxin m^{-3} was found in Finnish cow barns during milking and feeding (Louhelainen *et al.* 1997). Our results, revealing 10–20 times higher concentrations of air-borne endotoxin, may be explained by the use of Tween for extraction.

Sonesson *et al.* (1990) found 10–50 times more dust-borne 3-hydroxy fatty acids in settled indoor dust than could be accounted for by LAL-active endotoxin. Saraf *et al.* (1997) showed a strong correlation between LAL reactivity and the amount of C10–C14 hydroxy fatty acids in dusts. We found that the dust-contained fatty acids C14: iso3-OH and C17: iso 3-OH belonged to actinomycetes (Table 2), and possibly to some strains of *Bacillus licheniformis*, which emit lichenysin containing C18 and C16 3-hydroxy fatty acids (Yakimov *et al.* 1996).

It is concluded that some of the 3-hydroxy fatty acids found in dusts do not necessarily indicate the presence of *Limulus*-reactive LPS. Dust-contained signature fatty acids give useful information on accumulated bacterial contamination in an indoor environment where degradation of biomolecules is low (in dehydrated dust). The viable count assay may give the momentary information of the metabolically-active bacterial population, but it is less reliable for assessing the bacterial communities present earlier in the ecological succession of the emitting source.

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