Bacillus cereus Spores During Housing of Dairy Cows: Factors Affecting Contamination of Raw Milk

M. Magnusson,*¹ A. Christiansson,† and B. Svensson†

*Department of Agricultural Biosystems and Technology, Swedish University of Agricultural Sciences, SE-230 53 Alnarp, Sweden

†Swedish Dairy Association, Research and Development Department, SE-223 63 Lund, Sweden

ABSTRACT

The contamination of raw milk with *Bacillus cereus* spores was studied during the indoor confinement of dairy cattle. The occurrence of spores in fresh and used bedding material, air samples, feed, feces, and the rinse water from milking equipment was compared with the spore level in bulk tank milk on 2 farms, one of which had 2 different housing systems. A less extensive study was carried out on an additional 5 farms. High spore concentrations of >100 spores/L in the raw milk were found on 4 of the farms. The number of spores found in the feed, feces, and air was too small to be of importance for milk contamination. Elevated spore contents in the rinse water from the milking equipment (up to 322 spores/L) were observed and large numbers of spores were found in the used bedding material, especially in free stalls with >5 cm deep sawdust beds. At most, 87,000 spores/g were found in used sawdust bedding. A positive correlation was found between the spore content in used bedding material and milk ($r = 0.72$). Comparison of the genetic fingerprints obtained by the random amplified polymorphic DNA PCR of isolates of *B. cereus* from the different sources indicated that used bedding material was the major source of contamination. A separate feeding experiment in which cows were experimentally fed *B. cereus* spores showed a positive relationship between the number of spores in the feed and feces and in the feces and milk ($r = 0.78$). The results showed that contaminated feed could be a significant source of spore contamination of raw milk if the number of spores excreted in the feces exceeded 100,000/g.

Key words: *Bacillus cereus* spore, contamination source, dairy cattle, raw milk

INTRODUCTION

The spore-forming bacterium *Bacillus cereus* can survive pasteurization and is thus a limiting factor for the

shelf life of pasteurized milk (Griffiths, 1992). It is a potential food poisoning agent (Granum and Lund, 1997) because of this property. The problem with the presence of *B. cereus* spores in raw milk is most prominent during the grazing period (Slaghuis et al., 1997), when there is a risk of the teats being contaminated by soil (Christiansson et al., 1999).

Spores in milk can occur during the period when cows are kept indoors, but the sources of contamination have not been clearly identified. Possible sources of contamination that were considered are soil, feces, bedding, feed, air, and the milking equipment (Van Heddeghem and Vlaemynck, 1992). During the housing period, the cows are not in contact with the soil, as they are during the grazing period, but the teats can be contaminated by feces and bedding material. A positive correlation was reported between the *B. cereus* spore content in feed and in feces (Labots et al., 1965). Silage was considered a source of contamination. The same strains of *B. cereus* were found in silage and in feces (Torp et al., 2001), and of aerobic spore-formers in silage and in raw milk (te Giffel et al., 2002). The spore content of *B. cereus* in feed and feces was low (Slaghuis et al., 1997; Christiansson et al., 1999; Vaerewijck et al., 2001), although elevated spore contents were reported. Spent grain from the brewery contained more than 10^6 spores/g (Barkley and Delaney, 1980; Torp et al., 2001). The air in the barn was considered a source of contamination, but later studies have shown that the number of *B. cereus* spores in the air is too small for the air to be of major importance (te Giffel et al., 1995; Christiansson et al., 1999). Large numbers of aerobic spores were found in used bedding material (McKinnon and Pettipher, 1983), and bedding was suggested as a participant in the contamination route for *B. cereus* (te Giffel et al., 1995; Slaghuis et al., 1997). Labots et al. (1965) suggested that milking equipment could be a source of spore contamination, but later investigations found small amounts of spores in this equipment (McKinnon and Pettipher, 1983; Christiansson et al., 1999). The aim of this study was to evaluate the relationship between the occurrence of *B. cereus* spores in the housing environment of dairy cows and in raw milk, and to

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¹Corresponding author: madeleine.magnusson@ltj.slu.se

elucidate the routes of contamination using random amplified polymorphic DNA PCR (**RAPD-PCR**) fingerprinting of the bacteria.

MATERIALS AND METHODS

Farms and Experimental Design

Study 1. Two housing systems commonly used in Sweden were studied on farm 1 (Alnarp Dairy Research Station at the Swedish University of Agricultural Sciences). Both systems were in insulated buildings with 55 dairy cows in tie stalls and 65 in free-stall housing. The floor of the free stalls and tie stalls was covered with rubber mats. Free stalls were bedded twice weekly with 3 to 4 kg of sawdust and tie stalls were bedded twice daily, once with 1 to 2 kg of sawdust and once with 0.5 to 1 kg of chopped straw. The feed used in the tie-stall housing section was composed of grass silage, alfalfa silage, ensiled beet pulp, hay, straw, concentrate 1 (rapeseed cake, rapeseed meal, coconut, corn gluten, soy meal, and dried beet pulp), concentrate 2 (oats, barley, and dried beet pulp), and minerals. In the free-stall barn the cows were fed a TMR (grass silage, alfalfa silage, ensiled beet pulp, straw, and minerals), concentrate 1, and concentrate 3 (oats, barley, peas, and minerals). The cows were milked while in the tie stalls and cows in the free stalls were milked in a milking parlor. Premilking teat-cleaning was carried out in both systems using dry paper towels.

Samples were collected on 10 d during a 4-wk period in October and November for the analysis of *B. cereus* spores from the fresh and used bedding material, air, feed, feces, rinse water from the milking equipment after cleaning, and milk in the bulk tank. Visual inspection and scoring were made of the cleanliness of the udder and teats, and of the free stalls and tie stalls. The indoor temperature and humidity were recorded.

Study 2. A farm was investigated that previously had elevated spore contents in the bulk tank milk. Farm 2 had approximately 230 dairy cows kept in an uninsulated building with free stalls bedded with 30-cm-deep sawdust and without a concrete base. The cows were given a TMR (triticale, grass silage, straw, protein mix, and minerals) and concentrate (oats, barley, dried beet pulp, and purchased ready-made concentrate). The cows were milked in a milking parlor, and premilking teat-cleaning was carried out with dry paper towels.

Samples were collected for the analysis of *B. cereus* on 5 d during a 2-wk period in January from the used and fresh bedding material, air samples, feed, feces, water remaining in the water cups, rinse water, and milk in the bulk tank. Visual inspection and scoring were made of the cleanliness of the udder and teats, and

of the free stalls. The indoor temperature and humidity were recorded.

Isolates of *B. cereus* from the different sources were collected over a 3-mo period on the farm and compared by RAPD-PCR fingerprinting to establish identity among the isolates, and thereby the route of contamination.

Study 3. A smaller study was carried out on 5 additional farms, some with similar and others with different housing systems than farm 2: Farm 3 was a loose-housing system with 56 dairy cows in an uninsulated building with deep-straw bedding; farm 4 was an uninsulated building with 122 dairy cows housed in free stalls bedded with sand and without a concrete base; farm 5 was an insulated building with 300 dairy cows in free stalls bedded with 5 to 20 cm of sawdust on a concrete base; farm 6 was an insulated building with 150 dairy cows in free stalls bedded with 5 to 20 cm of sawdust on a concrete base; and farm 7 was a tie-stall system with 18 dairy cows in an insulated building, where the floor of the stalls was covered with rubber mats and bedded twice daily with chopped straw.

On each farm, milk and rinse water samples were collected on 4 to 7 d during a 14-d period in April. On 2 of the sampling days, 2 to 4 composite samples of used bedding material were collected. Milking routines on the farms were recorded.

Study 4: Feeding Experiment. Twelve cows in tie stalls were divided into 4 groups with 3 cows in each group. The cows were kept in a separate building at the Alnarp Dairy Research Station. Over 7 d, the cows were fed a dose of spores twice daily mixed into some dried beet pulp, with the purpose of achieving approximately 10,000, 100,000, and 1 million spores/g of feces in groups 1, 2, and 3, respectively. The largest dose was 4.5×10^{10} spores/cow provided twice daily. Groups 1 and 2 were fed 100 and 10 times fewer spores, respectively, than group 3. The control group did not receive any spores. Tie stalls had inlaid rubber mats and were bedded twice daily with approximately 2 kg of sawdust in the morning and 1 kg of chopped straw in the evening. The cows were milked while in the tie stalls, and teat-cleaning was carried out with dry paper towels.

Samples of milk and feces from each cow, and air samples from each group were collected during the morning milking on the last 3 d of the spore-feeding period. Visual inspection and scoring were made of the cleanliness of the udder and teats.

Sampling Procedures

The milk samples in studies 1 and 2 were taken from the bulk tank after the first milking. In study 3, the milk samples were taken from a full tank. The individ-

ual milk samples in the feeding experiment were collected with milk meters (Tru-test Ltd., Auckland, New Zealand). The samples of the rinse water from the milking equipment were collected before the milking. In studies 1 and 2, the samples were collected at 2 sampling points: at the outlet in the milk storage room (rinse water 1) and at the releaser (rinse water 2) following the circulation of 50 to 80 L of cold water for 2 to 5 min. In study 3, the residual rinse water remaining in the milking equipment from the last cleaning cycle was collected at the releaser just before milking. Air samples were collected with an RCS-sampler (Biotest Diagnostics, Soest, the Netherlands) equipped with blood agar strips. Samples were collected for 30 s at udder height close to the animals during milking and in the free stalls after milking. Samples of feed and unused bedding material were collected on each sampling day and analyzed as composite samples for each week. Used bedding material and feces were collected in the morning before the cleaning of randomly selected stalls. The bedding material was collected from the rear of the stalls as composite samples from 6 stalls for each sample, and mixtures with feces were avoided. In studies 1 and 2, the samples were taken from 10% of the stalls. The feces were collected from fresh dung piles from 10% of the cows as composite samples from 6 piles in each sample. In study 2, the samples of bedding material were taken at different depths (0, 10, 20, and 30 cm) from one randomly selected free stall on each sampling day. All fluid samples of 200 mL and solid samples of at least 100 to 200 g were frozen at -20°C , and the samples were thawed in cold water just before analysis. Fluid samples were analyzed within 3 d and solid samples within 14 d. Indoor temperature and relative humidity were recorded every hour during the sampling periods using a data logger (Multilogg, Danelko, Helsingborg, Sweden).

Cleanliness

Visual observation of udder and teat cleanliness was made on sampling days before the morning milking for 10% of the cows. The cleanliness of the udder, teats, and teat tips was scored on a 5-point scale (Christiansson et al., 1999), where score 1 was completely clean and score 5 was more than 50% of the area dirty. The cleanliness of all stalls was observed in the morning before they were cleaned and the number of contaminated stalls was noted. The stalls were recorded as being contaminated if a minimum area of 100 cm^2 was covered with feces.

Preparation of Spores

Large amounts of spores from *B. cereus* strain SMR 161 (obtained from the Culture Collection of the Univer-

sity of Gothenburg Sweden as CCUG 6514) were prepared by cultivation under controlled conditions in a fermentor (Chemoferm, Hågersten, Sweden). The growth medium was made up of 8.0 g of nutrient broth (Difco, Boule Nordic, Huddinge, Sweden), 5.0 g of yeast extract, 0.1 g of $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 0.2 g of $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, and 6.2 mg of MnCl_2/L . Cultivation was performed at 30°C with constant stirring and aeration for 46 h. The spores were harvested by centrifugation at $7,500 \times g$ for 10 min and were washed twice by suspension in physiological saline followed by centrifugation. The spores were resuspended in saline, diluted, and frozen at -20°C in aliquots with spore concentrations suitable for the experiment.

Microbiological Analyses

Milk and water samples of 50 to 100 mL were heat-treated in a water bath at 72°C for 5 min (Christiansson et al., 1997). The water samples were filtered through a membrane filter (LKB-Sartorius AB, Sundbyberg, Sweden) having an $0.8\text{-}\mu\text{m}$ pore size (11404-47-ACN) using a filtration apparatus equipped with a sterilizable filter support and funnels (Sartorius SM16831). The milk samples were treated with trypsin and Triton X-100 (Christiansson et al., 1997). Following filtration, the filters were rinsed with 100 mL of sterile water at 55°C . The filters for *B. cereus* counts were placed on the surface of blood agar plates [blood agar base No. 2 (Oxoid), 10 ppm of polymixin B sulfate (Sigma Chemical, St. Louis, MO), and 5% bovine defibrinated blood]. The plates were incubated aerobically at 20°C for 48 h, and typical colonies of *B. cereus* showing a zone of hemolysis were counted. With this procedure, spores of both mesophilic and psychrotrophic *B. cereus* were counted. When necessary, confirmation of identity was made by phase-contrast microscopy and plating on MYP agar (mannitol-egg-yolk-phenol red agar; Mossel et al., 1967), and by biochemical typing using an API 50 CHB/20E system (bioMérieux, Marcy-Étoile, France).

For solid samples (feed, feces, sawdust), 25 g was added to 225 mL of sterile peptone water (2 g of peptone/L, and 0.1 g of Tween 80/L) in a Colworth Stomacher bag (Seward Limited, London, UK) and then homogenized twice in a Colworth Stomacher for 30 s each. From the Stomacher bag, 100 mL of liquid was transferred to a sterile 100-mL cylinder. Following 2 min of sedimentation, 10 mL was collected at the 50-mL mark and transferred to a test tube, which was heat-treated at 72°C for 5 min before the analysis for the presence of *B. cereus* spores. Serial 10-fold dilutions were surface-plated in duplicate for the determination of *B. cereus* spores on blood agar plates incubated aerobically at 20°C for 48 h. Blood agar strips from the air sampler were incubated

aerobically at 20°C for 48 h for counting *B. cereus* colonies.

Fingerprints with RAPD-PCR

Bacillus cereus colonies were collected randomly from each of the filters and plates. The colonies were checked for purity by streaking on plate count agar and stored frozen at -80°C in Nutrient Broth (Difco) with 20% glycerol. The DNA template for PCR-amplification was extracted from pure cultures according to the method described by Nilsson et al. (1998). The RAPD-PCR analysis was performed using primer (5' CCGAGTCCA 3'; Pharmacia Sweden, Uppsala, Sweden) and AmpliTaq DNA polymerase (PerkinElmer, Norwalk, CT). The PCR products were separated on a 1.5% agarose gel, visualized on a UV transilluminator, and photographed and scanned. The GelCompar 4.0 software (Applied Maths, Kortrijk, Belgium) was used to collect the densitometric traces of the electrophoretic patterns, to normalize the band positions relative to molecular weight standards, and to conduct numerical analyses. Cluster analyses based on Pearson correlation coefficients were performed between the densitometric traces of the PCR fingerprints. Linkage was performed according to the method of Ward (1963). The evaluation of RAPD patterns with this software was standardized and enabled data from different sampling occasions to be combined into dendrograms. It permitted comparison between RAPD fingerprints from isolates sampled over long time periods (Svensson et al., 2004).

Statistical Analyses

Statistical calculations were performed using SYSTAT version 9.0 (SPSS, 1999). Log-transformed values for *B. cereus* counts were generally used for statistical analyses by Pearson correlations and ANOVA. Differences between groups in the feeding experiment were tested by Tukey's test. In some of the studies, several results of the bacteriological analyses were below the detection level. In these cases, and when log-transforming did not show a normal distribution, nonparametric analyses were used, such as the Wilcoxon signed rank test and the Mann-Whitney *U*-test. To rank values below the detection level, the results were assigned half the detection level. Comparison between ratios of different strains in different environments was done with Fisher's exact test.

RESULTS

Study 1

The indoor temperature and relative humidity during the sampling period were 15.6°C (range, 11.7 to 18.5°C)

and 72% (range, 38 to 85%) in the tie stall, respectively. Corresponding values in the free-stall barn were 14.4°C (range, 9.0 to 17.7°C) and 85% (range, 51 to 94%), respectively. The tie stalls were more contaminated ($56.4 \pm 2.4\%$) than the free stalls ($24.5 \pm 1.8\%$), yet the cows were fairly clean in both systems. The cleanliness scores of the udder, teats, and teat tips were 1.6 ± 0.12 and 1.6 ± 0.10 for contamination with feces, and 2.0 ± 0.12 and 2.3 ± 0.11 for contamination with feces and bedding in tie stalls and free stalls, respectively.

The *B. cereus* spore contents in the milk obtained from the cows in both systems were very small, often smaller than the detection level (10 spores/L of milk; Table 1). The greatest observed spore content in the milk was 39 spores/L. The rinse water contained few spores, often below the detection level. Samples from the environment, the feces, and the feed had few spores. The *B. cereus* concentration varied in the air samples, with the greatest value being 200 spores/m³. Spores were not detectable in either the sawdust or the straw (Table 2) but could be found in the used bedding (Table 1). The largest number of spores found in the used bedding material was 1,900 spores/g. There were no differences in spore contents between the 2 housing systems in any of the traits shown in Table 1 (Wilcoxon signed rank test, $P > 0.20$). Of the concentrates, only concentrate 1 had detectable level of spores, but not more than 150 spores/g (Table 2). The only feed substance that consistently contained detectable spore counts was the alfalfa silage, up to 550 spores/g.

Study 2

The average indoor temperature and relative humidity during the sampling period on farm 2 in the free-stall barn were 2.1°C (range, -6.2 to 7.7°C) and 94% (range, 75 to 99%), respectively. Only $10.8 \pm 0.4\%$ of the free stalls were contaminated, and that was less than found in study 1 ($P < 0.001$). The cows appeared to be clean. The cleanliness scores of udder, teat, and teat tips were 1.1 ± 0.05 for contamination with feces, and 2.6 ± 0.09 for contamination with feces and bedding material; the teats and teat tips were especially contaminated with sawdust. The *B. cereus* spore content in the milk was 81 ± 17 spores/L and was significantly larger than found on farm 1 (Table 3). In comparison with the results of study 1, greater spore concentrations of 60 ± 19 and 194 ± 43 spores/L were found in rinse water 1 and 2, respectively (Mann-Whitney *U*-test, $P < 0.005$). The spore content in the air samples was larger than found in study 1, with a maximum value of 500 spores/m³. The feed contained <50 spores/g, and thereby the spore content in the feces was small (<100 spores/g).

Table 1. Presence of *Bacillus cereus* spores in bulk tank milk and in environmental samples on farm 1 in study 1

Sample	Free stall		Tie stall		Number neg. (total) ²	Limit of detection
	Mean ¹	Maximum	Mean	Maximum		
Milk, per L	<10	14	11	39	6 + 6 (10 + 10)	10
Rinse water 1, per L	12	41	10	54	7 + 9 (10 + 10)	10
Rinse water 2, per L	10	48	7	14	8 + 7 (10 + 10)	10
Air during milking, per m ³	<50	50	60	200	7 + 6 (10 + 10)	50
Air in lying area, per m ³	<50	100	—	—	8 (10)	50
Feces, per g	<50	50	38	100	8 + 7 (10 + 10)	50
Used bedding material, per g	103	300	313	1,900	5 + 1 (10 + 10)	50

¹Arithmetic mean calculated by setting each value below the detection level equal to half the value of the detection level.

²Number of negative samples in free stalls and in tie stalls (of total number of samples).

The water remaining in the water cups had large spore contents (>1,500 spores/L).

The fresh bedding material contained <50 spores/g, but used bedding material had a considerably greater spore content than found in study 1 (Mann-Whitney *U*-test, $P < 0.001$). Vast differences were found in the spore contents obtained from the samples of bedding material in the surface layer (900 to 62,000 spores/g). Litter was added to the free stalls twice weekly and on different days in different sections of the free-stall barn. Following the addition of fresh material, the spore contents decreased markedly. Samples taken from different depths of the bed (from the surface, 10, 20, and 30 cm depths) showed that more spores were found in the surface layer and at a 10-cm depth ($21,500 \pm 7,800$ spores/g) than at the 2 bottom layers (850 ± 380 spores/g; $P = 0.012$). In the 2 top layers the spore content varied between 2,300 and 65,000 spores/g and in the 2 bottom layers between <50 and 3,600 spores/g.

RAPD-PCR. Preliminary studies on RAPD fingerprints of isolates from different sources on farm 2 indi-

cated that identical RAPD patterns were found in isolates taken from most of the sources; for example, the same strain was found in used bedding material, water, air, and milk. Nevertheless, these isolates were not always from the same sampling day (i.e., interpretation of the contamination routes was not straightforward). Furthermore, there were several strains with different RAPD patterns that occurred in a similar manner. We decided to extend the sampling period for RAPD-PCR over 3 mo based on the hypothesis that for important contamination routes (i.e., from one source to another) there could be a temporal correlation. Thus, a larger ratio between pairwise identical isolates from 2 sources (in any RAPD group) and all isolates could be expected on the same sampling days if there was a direct mechanism of transfer in comparison with the ratio between 2 sources with more random or less frequent transfer. In total, 125 isolates of *B. cereus* from the different sampling sources on farm 2 were examined by RAPD-PCR. The isolates were divided into 22 RAPD groups, each considered to consist of isolates with identical fingerprints. As noted in the preliminary trial, many isolates having the same RAPD group were found in the bedding material, rinse water, and air samples, as well as in the milk, although they were not always isolated on the same sampling day. In Table 4, the relationship between isolates from 2 sources is described as a ratio between the sum of all pairwise identical isolates found on the same day and the total sum of all identical isolates from the 2 sources. Because isolates were not always found in all sources on each sampling day, the numbers of isolates from the different sources were not the same. Pairwise comparisons between ratios were performed.

A larger proportion of time-related identical strains was found when comparing the bedding material and milk than when the air and milk were compared (Table 4; $P = 0.01$). No significantly different ratio of strains from the bedding material and milk than from the bed-

Table 2. Presence of *Bacillus cereus* spores in fresh litter and feed on farm 1, in study 1

Sample	Mean, ¹ spores/g	Maximum, spores/g	Number neg. (total) ²
Sawdust	<50	<50	4 (4)
Chopped straw, litter	<50	<50	4 (4)
Hay	<50	50	3 (4)
Straw, feed	<50	<50	4 (4)
Concentrate 1, tie stall	81	150	1 (4)
Concentrate 1, free stalls	50	100	2 (4)
Concentrate 2, tie stall	<50	<50	4 (4)
Concentrate 3, free stalls	<50	<50	4 (4)
Grass silage	75	150	2 (4)
Alfalfa silage	413	550	0 (4)
Ensiled beet pulp	<50	<50	4 (4)
TMR, free stalls	138	350	1 (10)

¹Arithmetic mean calculated by setting each value below the detection level (<50) equal to half the value of the detection level.

²Number of negative samples (of total number of samples).

Table 3. Presence of *Bacillus cereus* spores in bulk tank milk, environmental samples, feed and fresh litter on farm 2 in study 2

Sample	Mean ¹	Maximum	Number neg. (total) ²	Limit of detection
Milk, per L	81	137	0 (5)	10
Rinse water 1, per L	60	129	0 (5)	10
Rinse water 2, per L	194	322	0 (5)	10
Air, lying area, per m ³	145	350	1 (5)	50
Air, eating area, per m ³	330	500	0 (5)	50
Air, milking area, per m ³	275	500	1 (5)	50
Feces, per g	<50	100	17 (20)	50
Used bedding, surface of bed, per g	17,800	62,000	0 (18)	50
Sawdust, fresh, per g	<50	50	3 (5)	50
Concentrate, per g	<50	<50	2 (2)	50
TMR, per g	<50	<50	4 (4)	50
Drinking water, per L	>1,500	>1,500	0 (5)	10

¹Arithmetic mean calculated by setting each value below the detection level equal to half the value of the detection level.

²Number of negative samples (of total number of samples).

ding material and air was found ($P = 0.61$), indicating that the sawdust was the source of contamination of both milk and air on this farm. The ratio of time-related strains in rinse water 1 and bedding material was larger than in rinse water 1 and milk ($P = 0.04$). These results indicated that the bedding material was the primary source of contamination of the milk. The same relations were not found for rinse water 2 (Table 4).

Study 3

Results of the analyses of samples taken from farms 3 to 7 are summarized in Table 5. The content of *B. cereus* spores in the milk from all the farms in study 3 was larger (Mann Whitney *U*-test, $P < 0.001$) than that obtained from farm 1 in study 1. The lowest spore content in the milk, 33 ± 18 spores/L, was found on farm 4, where sand was used as bedding in the free stalls. The 2 farms using sawdust as the bedding material had

high spore contents in the milk (63 ± 15 and 269 ± 111 spores/L). Farm 5 had the highest spore content in the milk; 3 samples had more than 400 spores/L, and the largest value was 1,100 spores/L. Even farm 7 with tie stalls had an elevated spore content in the milk (124 ± 18 spores/L). Farm 3 had 36 ± 7 spores/L in the milk, and the spore content in the straw obtained from the deep-straw bedding varied greatly between 400 and 5×10^6 spores/g; however, only few samples had very high spore contents. Farms 5 and 6 with sawdust had high spore contents in used bedding material; the largest number of spores found in an individual sample was 87,000 spores/g.

All Farms

The relationship between the spore content of the bedding material and the milk from all farms included in these 3 studies is summarized in Figure 1. An increase in the spore contents of the bedding material increased the risk for elevated spore concentrations in the bulk tank milk ($r = 0.72$; $P < 0.001$). The coefficients of correlation between the spores in the milk, residual rinse water, or rinse water 2 and in bedding material on farms with elevated spore content (i.e., farms 2 to 7) were calculated on data obtained from sampling days when samples from all 3 sources were available. The 2 extreme values found for the bedding material from farm 3 were considered as outliers (Figure 1) and excluded from the calculation. A positive correlation of spore content was found between the bedding material and milk ($r = 0.75$; $P < 0.001$), and between the bedding material and residual rinse water ($r = 0.57$, $P = 0.013$), but not between the residual rinse water and milk.

Table 4. The ratios between the sum of all pairwise time-related (in both sources on the same sampling day) identical isolates as demonstrated by random amplified polymorphic DNA fingerprinting found in 2 different sources, and the total number of identical isolates found in the same sources independent of sampling day on farm 2 in study 2¹

Comparison	Milk	Rinse water 1	Rinse water 2	Air
Bedding	11/44 ^A	8/25 ^{DE}	4/34 ^H	10/32 ^I
Milk	—	2/26 ^{CD}	9/35 ^{BF}	0/20 ^{ABI}
Rinse water 1	—	—	7/9 ^{CEFGH}	2/9
Rinse water 2	—	—	—	4/21 ^G

^{A-I}Ratios with one source in common are tested pairwise. Ratios with the same letter of superscript differ in pairwise comparisons ($P < 0.05$).

¹Bacterial isolates were selected randomly on each sampling day from the 5 sources.

Table 5. Presence of *Bacillus cereus* spores in milk, residual rinse water, and used bedding material on farms 3 to 7 in study 3

Farm no.	Bedding	Milk, spores/L			Residual rinse water, spores/L			Used bedding, spores/L		
		Mean	Max.	(n) ¹	Mean	Max.	(n) ¹	Mean ²	Max.	(n)
3	Deep-straw bed	36	60	(5)	40	59	(5)	2,400,000 ³	5,000,000	(4)
4	Sand	33	80	(4)	17	42	(3)	213	363	(2)
5	Sawdust	269	1,100	(10)	51	103	(9)	16,150	23,230	(2)
6	Sawdust	63	120	(7)	27	61	(6)	20,130	33,750	(2)
7	Straw	124	180	(5)	39	84	(5)	8,200	14,350	(4)

¹(n) = number of samples.²Mean of the spore content for all sampling occasions (n occasions). At each occasion the spore content was based on the arithmetic mean of the spore content of bedding material in different sections (2 to 4) of the lying area.³Two samples >1 million/g, the rest only 400 to 19,000/g (mean 4,000).

Study 4: Feeding Experiment

On the sampling days, there were no significant differences in the cleanliness of the udder, teat, and teat tips between the groups. Differences between groups were obtained for the spore contents of the feces ($P < 0.001$), approximately 1 log increase for each group. Differences between groups were found for the spore content of milk ($P < 0.001$; Figure 2). The control group had unexpectedly elevated milk spore contents (87 ± 26 spores/L). Upon PCR analysis of isolates from this group, DNA fingerprints were found that did not correspond to the *B. cereus* strain SMR 161 given in the feed. This was not observed to the same extent in the other groups. Most likely, the elevated spore contents in the control group milk were due to contamination from the milking equipment, because the same *B. cereus* strains could be found in the rinse water. Therefore, the spore

concentrations for all the observations were corrected for the background contamination and the results of the control group were excluded in the comparison between the feces and milk spore contents (Figure 3). For groups 1 to 3, a significant correlation was found between the spore contents in the feces and the milk ($r = 0.78$, $P < 0.001$). There was an elevated risk of having increased spore levels in the milk (>100/L) if the spore content of the feces exceeded 10,000 to 100,000 spores/g.

The spore content in the air differed between the groups, and up to 1,750 spores/m³ of air were detected in group 3. The correlation coefficient between the spore content in the milk and in the air was 0.61 ($P = 0.001$), and between the spore content in the feces and in the air was 0.67 ($P < 0.001$).

DISCUSSION

The results indicated that there was active growth and sporulation of *B. cereus* in the bedding material, because the bedding material remained in the free stalls for a long period of time. New bedding was added twice weekly in the 30-cm-deep beds on farm 2, and on farms 5 and 6 with 5- to 20-cm-deep sawdust beds, littering occurred every 10th and 14th day, respectively. The spore content noted in the beds on farm 2 before and after littering indicated that the management of free stalls might be important in controlling the presence of *B. cereus* spores in the milk. Adding new bedding material daily or a frequent total replacement of the sawdust bed may reduce the spore content in the beds. On farm 1, the small amount of bedding in tie stalls and free stalls, and the short time the bedding material remained did not allow bacterial growth to large numbers. Farm 4, using sand as bedding material, had a low spore content in the sand and in the milk, even though littering took place only once monthly. Inor-

Figure 1. *Bacillus cereus* spore levels in used bedding material and in bulk tank milk on farms 1 to 7 (n = 40).

Figure 2. Effect of feeding cows with *Bacillus cereus* spores on the spore content of feces and milk from individual cows in study 4 (three cows in each group). Group 0: no spores fed. Groups 1 to 3: spores added to the feed. ^{a-d}Groups with different superscripts differ ($P < 0.05$).

ganic bedding such as sand contained fewer bacterial counts of gram-negative bacteria, coliforms, and *Klebsiella* species than did sawdust and chopped straw (Hogan et al., 1989). It is possible that sand could be a less suitable environment for the growth of *B. cereus*. On farm 7, the tie stalls were bedded with straw and cleaned and bedded every day, but still large spore contents were found in the milk. A possible cause, according to the farmer, could be that straw of poor quality was used. The spore content in used bedding material was considerably higher than was found in the tie-stall system on farm 1. The milking also occurred in the tie stalls.

Figure 3. Effect of increasing the *Bacillus cereus* spore content in feed on the spore level in the feces and the milk in study 4. Groups 1 to 3 included $n = 27$.

It was not possible to explain the differences between the spore content in milk on the 6 farms by the different milking routines, but they probably did have an effect (Magnusson et al., 2006). Farm 3, with a deep-straw bed, had extremely large spore contents in some samples of used bedding material, whereas other samples had comparatively small spore contents. Good milking routines by washing the udder with lukewarm water from a hose and drying with paper toweling probably contributed to the small spore content found in milk. Another explanation could be that long straw might not adhere to the udder to the same extent as sawdust.

The results of the present investigation showed that when the concentration of *B. cereus* spores in the bedding exceeded 10,000/g, there was an increased risk of having unwanted spore contents in the milk (>100 spores/L). Large numbers of aerobic spores were observed in the bedding (McKinnon and Pettipher, 1983; Slaghuys et al., 1991).

The spore content of the rinse water was elevated on farm 2. Rinse water 2 was more contaminated than rinse water 1 and was even more contaminated than the residual rinse water on farm 5, which also had a large spore content in the milk. Because rinse waters 1 and 2 were the same circulated water sampled at different places, additional contamination of rinse water 2 must have occurred in close vicinity to the sampling point, possibly the valve, yet visual inspection did not reveal any residues. On farm 2, rinse water 1 most likely gave a better reflection of the total contamination status of the entire milking equipment, because sampling was performed at the outlet in the milk tank room. It was not possible to determine whether the bedding was the only contamination source, or whether the milk was also contaminated by the milking equipment. The

increased spore content observed in the rinse water could be caused partly by the milking equipment, but the results of the RAPD-PCR analyses did not indicate this as the major route of contamination with respect to rinse water 1 passing through the entire milking equipment. Nevertheless, the results for rinse water 2 were different and might reflect some contamination in the equipment. On the other hand, during study 4 spores with RAPD-PCR fingerprints differing from the *B. cereus* strains fed to the cows were found in the milk. These spores likely originated from the milking equipment because the strain also was found in the rinse water. No bedding samples were taken in study 4 because the small amount of bedding material and the bedding routines were the same as used in the tie stalls in study 1. Because of the short turnover time of the bedding material, extensive growth would not have been possible. The amount of spores in feces would far outnumber any spores originating from growth in the bedding material. *Bacillus cereus* spores are more designed to adhere to surfaces compared with other bacterial spores and vegetative cells (Rönner et al., 1990). Poor cleaning and disinfection may lead to greater numbers of spores present in the milk.

The water remaining in the drinking cups on farm 2 contained relatively large numbers of spores. This might have been caused by feed residues and subsequent bacterial growth in the cups, but the small amounts noted would hardly contribute to the presence of spores in the milk.

The *B. cereus* content of the air was generally low. An elevated number of spores was found during the feeding experiment (study 4), but air would still only contribute small amounts of spores in the milk at milking. These results agreed with those of other investigations (te Giffel et al., 1995; Christiansson et al., 1999). The RAPD-PCR analyses in study 2 confirmed that the air does not appear as an important source of milk spore contamination.

The *B. cereus* spore content in concentrate, TMR, and silage used in studies 1 and 2 were low, as in earlier studies (Slaghuis et al., 1997; Christiansson et al., 1999), and consequently, so was the spore content in feces; it did not exceed 100 spores/g. The feeding experiment (study 4) results showed that there was a positive correlation between the spores in the feed and in the feces, supporting Labots et al. (1965). The presence of more than 10,000 to 100,000 spores/g of feces could reduce milk quality.

In previous investigations, silage was considered a source of *B. cereus* contamination (Torp et al., 2001; te Giffel et al., 2002). Group 3 in the feeding experiment was fed with the largest amount of spores, that is, 9×10^{10} spores/d; this number of spores would be equivalent

to that ingested by cows fed 30 kg of reduced hygienic quality silage with a spore content of 3×10^6 spores/g. The spore content of the feces from cows in this group was 410,000 spores/g, suggesting that a considerable portion of the spores was unaffected by passing through the digestive tract (Van Heddeghem and Vlaemynck, 1992). Feeding a large quantity of silage having a *B. cereus* spore content of >100,000 spores/g could generate a spore content of >10,000 spores/g in the feces and may be a risk to milk quality. Under similar circumstances, feeding silage of inferior quality will lead to large numbers of clostridial spores in milk (Stadhouders and Jørgensen, 1990).

In conclusion, large numbers of *B. cereus* spores occurred in milk from dairy cows in certain housing systems during confinement periods. The most important contamination source found was the bedding material, which contained large numbers of spores and contaminated the milk via contaminated teats. Large spore numbers were found in deep sawdust beds. Further work is needed to investigate whether different management routines or different bedding material can reduce the bacterial growth of *B. cereus*. Cows experimentally fed *B. cereus* spores showed that spore-contaminated feed could be a source of contamination, via feces and contaminated teats. In addition, where the cleaning procedures are meager, the milking equipment could contribute to contamination of the milk.

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