***Bacillus cereus* in Free-Stall Bedding**

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ABSTRACT

To increase the understanding of how different factors affect the bacterial growth in deep sawdust beds for dairy cattle, the microbiological status of *Bacillus cereus* and coliforms in deep sawdust-bedded free stalls was investigated over two 14-d periods on one farm. High counts of *B. cereus* and coliforms were found in the entire beds. On average, 4.1 log₁₀ *B. cereus* spores, 5.5 log₁₀ *B. cereus*, and 6.7 log₁₀ coliforms per gram of bedding could be found in the upper layers of the sawdust likely to be in contact with the cows' udders. The highest counts of *B. cereus* spores, *B. cereus*, and coliforms were found in the bedding before fresh bedding was added, and the lowest immediately afterwards. Different factors of importance for the growth of *B. cereus* in the bedding material were explored in laboratory tests. These were found to be the type of bedding, pH, and the type and availability of nutrients. Alternative bedding material such as peat and mixtures of peat and sawdust inhibited the bacterial growth of *B. cereus*. The extent of growth of *B. cereus* in the sawdust was increased in a dose-dependent manner by the availability of feces. Urine added to different bedding material raised the pH and also led to bacterial growth of *B. cereus* in the peat. In sawdust, a dry matter content greater than 70% was needed to lower the water activity to 0.95, which is needed to inhibit the growth of *B. cereus*. In an attempt to reduce the bacterial growth of *B. cereus* and coliforms in deep sawdust beds on the farm, the effect of giving bedding daily or a full replacement of the beds was studied. The spore count of *B. cereus* in the back part of the free stalls before fresh bedding was added was 0.9 log units lower in stalls given daily bedding than in stalls given bedding twice weekly. No effect on coliform counts was found. Replacement of the entire sawdust bedding had an effect for a short period, but by 1 to 2 mo after replacement, the

counts of *B. cereus* spores in the beds had increased about 2 log units and were as high as they were before bed replacement. Therefore, free-stall management could, to a limited extent, reduce the content of *B. cereus* spores in the beds by daily bedding and entire bed replacement.

Key words: *Bacillus cereus* spore, bedding material, dairy cattle, sawdust

INTRODUCTION

Counts of bacteria and spores in bedding for dairy cattle have been found to correlate with the counts found in the bulk tank milk (Hogan et al., 1988; Magnusson et al., 2007). *Bacillus cereus* spores can survive pasteurization and are one of the most important factors limiting the shelf life of pasteurized milk (Griffiths, 1992). *Bacillus cereus* is occasionally also a cause of clinical mastitis (Jones and Turnbull, 1981). Few studies have dealt with the presence of bacterial spores in relation to the bedding material (Slaghuis et al., 1991; Bernard et al., 2003).

The bedding material in the lying area always contains various amounts of different bacteria to which the cow's udder and teats are exposed. The rate of intramammary environmental coliform infections in dairy cows is correlated with the number of bacteria on the teat end (Hogan et al., 1989). The number of bacteria on the teat end is closely related to the number of bacteria in the bedding (Rendos et al., 1975). Using inorganic bedding such as sand has been shown to result in the presence of lower counts of environmental mastitis pathogens in the bedding than those found when using organic bedding such as sawdust for the cows (Fairchild et al., 1982; Hogan et al., 1989). The amounts of feces and urine and the DM content of the bedding are also of importance for the proliferation of bacteria (Zehner et al., 1986; Zdanowicz et al., 2004). Using lime and alkaline or acidic bedding conditioners affects the pH of the bedding material and has been shown to reduce the bacterial counts in organic bedding (Hogan and Smith, 1997; Hogan et al., 1999, 2007).

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Deep-bedded free stalls are considered to provide good cow comfort. Dairy cows prefer to lie on softer surfaces (Herlin, 1997; Tucker et al., 2003), and fewer leg injuries were found with deep-bedded free stalls than when mattresses were used (Weary and Taszkun, 2000; Wechsler et al., 2000). In deep-bedded free stalls, the bedding materials remain in the stalls for a longer period than in free stalls with rubber mats or mattresses bedded with a thin layer of bedding material. Deep sawdust beds in free-stall housing have been shown to be a potential contamination source of *B. cereus* spores in raw milk (Magnusson et al., 2007). The knowledge about the growth and sporulation of *B. cereus* in bedding for dairy cows is limited, and there are no recommendations for suitable management procedures for deep-bedded free stalls to ensure that there are low spore counts in the raw milk.

The objectives of this study were 1) to increase the understanding of how different factors affect the growth of *B. cereus* in deep sawdust beds, and 2) to evaluate the effect of modifying the management of deep sawdust beds in free stalls to reduce the content of *B. cereus* spores and coliforms in the beds.

MATERIALS AND METHODS

The microbiological status of *B. cereus* and coliforms in deep sawdust-bedded free stalls was investigated on one farm. The effect of the daily addition of bedding or the entire replacement of the sawdust beds was studied in an attempt to reduce the bacterial concentration in the beds. In addition, some exploratory laboratory tests were carried out to study the effects of different factors of importance for the bacterial growth of *B. cereus* in the bedding material.

The Farm

Three experiments (studies 1, 3, and 4) were carried out during the indoor confinement periods (August to April) on a farm previously investigated and observed to have elevated *B. cereus* spore contents in the bulk tank milk; up to 400 spores/L milk had been found. The major spore contamination source was found to be the sawdust beds in the free stalls (Magnusson et al., 2007). The farm had about 300 Swedish Holstein-Friesian cows housed in an uninsulated building with free stalls [130 × (225 to 250) cm] bedded with 30-cm-deep sawdust over a gravel base. There was no concrete flooring in the cubicles. The barn had been in use for 4 yr, and the sawdust in the free stalls was replaced once a year. The most recent replacement had taken place about 1 yr before these studies. The free stalls were cleaned thrice daily, and approximately 75 L of fresh sawdust per free

stall was added twice weekly. The alleys were scraped by automatic scrapers 5 to 7 times per day. The cows were milked 3 times a day and the premilking teat-cleaning routine was changed during the first study. In study 1, during the first study period, the teats were cleaned with dry paper towels; during the second period and in the following studies, the teats were sprayed with a soap solution (Hamra soap, DeLaval, Drongen, Belgium) before being dried with the paper towels. The sawdust was stored indoors and ventilated with non-heated air during studies 1 and 3, and stored outdoors covered by a roof during study 4 until needed.

Study 1: The Presence of *B. cereus* and Coliforms in Bedding

An investigation into the occurrence of *B. cereus* (spores and total counts) and coliforms in the free-stall beds was carried out over two 14-d periods in March and April, with 1 mo between periods. Bedding samples were collected on 6 and 7 sampling occasions during periods 1 and 2, respectively. They were collected at 6 different sampling points in each free stall; in 3 locations that were 15 to 50 cm from the curb of the stall, and in 3 locations at the front part located 145 to 175 cm from the curb. The samples were collected at the surface and at 3 depths of the bedding, with the first sampling taking place immediately after the fresh bedding had been added to the free stalls on d 0 and thereafter on d 2, 3, and 4 after bedding was added, respectively. The number of samples collected on the different days, locations, and depths are shown in Table 1. A total of 198 bedding samples were collected and analyzed for the presence of *B. cereus* and coliforms. Samples of bedding at the surface and at the 10-cm depth were taken as composite samples for each sampling place from 10 randomly selected stalls on each sampling occasion. Samples from 20- and 30-cm depths were taken from 1 randomly selected free stall on each sampling occasion. Twelve of the bedding samples were analyzed for DM and 43 for water activity (a_w). Samples of fresh sawdust before use were analyzed for bacteria and spore counts, DM, and a_w . The temperature was registered in 1 bed at the surface and at 3 depths in 6 locations. Milk samples from the bulk tank milk were collected on 4 and 6 occasions, respectively, during each 14-d period, and analyzed for the presence of *B. cereus* spores.

Study 2: Laboratory Experiments

The laboratory experiments were carried out in plastic containers (17 × 23 × 18 cm) with lids. Defined amounts (wt/wt) of bedding material, water, urine, and

Table 1. Number of sampling occasions of bedding material according to days after the addition of fresh bedding (d 0, 2, 3, and 4)¹

Depth	d 0		d 2		d 3		d 4	
	Front	Back	Front	Back	Front	Back	Front	Back
Surface	3	3	3	3	3	3	4	4
10 cm	3	3	—	—	1	1	4	4
20 cm	3	3	—	—	1	1	4	4
30 cm	2	2	—	—	—	—	2	2

¹Samples were obtained from different locations (front and back) in the free stalls and at different depths of bedding during study 1.

sterilized feces were carefully mixed in each container, and *B. cereus* spores were added to an amount of 3 log₁₀ spores/g. The containers were incubated at 30°C for 6 to 8 d. Samples for analyses of *B. cereus* spores and the total counts of *B. cereus* were taken daily for the first 4 d and then at d 6, 7, or 8. Samples were collected at the 5-cm depth in 1 location per sampling day and at different locations on each day in a predetermined pattern. The DM, a_w, and pH of samples from the last sampling day were evaluated.

Urine and fresh feces were collected from the dairy cows and studied with respect to their serving as a source of nutrients for *B. cereus*. The feces was diluted with water 1:1 (wt/wt) and sterilized at 125°C for 30 min before use. The different bedding materials tested were chopped straw, sawdust, peat, sand, mixtures of chopped straw and peat, and mixtures of sawdust and peat. Different batches of bedding material were used in the experiments.

Bedding Materials. Two experiments using different single bedding materials and one experiment using mixed bedding materials were carried out. The DM content was kept at 30% except for sand, which had a DM of 90%, and the content of feces was kept at 7%. These standard conditions in the experiment were set to reproduce the natural conditions in the free-stall bedding. Temperatures of 30°C could occur when the cows lay on the beds, a DM content of 30% could be found in wet parts of the beds, and the 7% DM content of feces in the final mixture was chosen by comparison of the color of the sawdust mixed with different proportions of feces compared with that of the used bedding removed from the free stalls.

Feces. The effect of different amounts of feces in the sawdust (1, 2, 5, 7 and 10%, respectively) was studied in one experiment; the DM was kept at 30%.

Urine. The effect of urine as the only nutrient and fluid in different bedding materials was studied in one experiment; the DM was kept at 30%, except for sand (90%).

DM. The effect of different percentages of DM in the sawdust was studied in one experiment. Sawdust hav-

ing a DM content of 45, 40, 30, and 20%, respectively, was used. The content of feces was kept at 7%.

Water Activity. Desorption and adsorption curves of the DM contents vs. the a_w in the sawdust were evaluated to obtain an understanding of the DM needed to prevent the growth of *B. cereus* in the sawdust. Sawdust with an original DM of 40% was dried slowly at room temperature (approximately 22°C) to 91.4% DM. During the drying period, samples were taken for determination of a_w. Thereafter, water was added to a calculated DM of 85, 80, 75, 70, 60, 40, and 30%, respectively. Samples were taken for the determination of the DM and a_w at each calculated DM level.

Study 3: Bedding Frequency

This experiment was carried out over a 4-wk period in August in a group of 140 lactating dairy cows. The same free stalls that were studied in study 1 were used. One row of 14 free stalls in the center of the barn was bedded with 30 L of sawdust per free stall once daily. The opposite row with 14 free stalls was bedded with 75 L of sawdust per free stall twice a week. The fresh bedding was added particularly to the back part of the free stalls, where the udder was likely to be in contact with the bedding material. The concentration of *B. cereus* (spores and total counts), and coliforms in the beds was studied. Samples of sawdust in the beds were collected on 6 occasions during the last 2 wk of the experiment. They were collected at the back of the free stall, 50 cm from the curb of the stall, and at the front, 145 cm from the curb. The samples were collected on the same days in both treatments: at the surface and at the 10-cm depth immediately after the bedding was given on d 0 (n = 3) and before the fresh bedding had been added on d 1, and d 3 or 4 (n = 3), respectively. At a depth of 20 cm, samples were taken on 4 of the sampling occasions. Samples of bedding at the surface and at the 10-cm depth were taken as composite samples from 10 stalls. Samples from 20- and 30-cm depths were taken from 1 randomly selected free stall on each

sampling occasion. Four samples of sawdust before use were analyzed for bacteria and spore counts.

Study 4: Entire Bed Replacement

The study was carried out between August and November in a group of 140 lactating dairy cows. The same free stalls that were studied in study 1 were used. One row with 14 free stalls in the center of the barn was completely cleaned, removing all the old bedding and replacing it with fresh sawdust. Thereafter, fresh sawdust was added to the free stalls twice weekly. The bacterial growth of *B. cereus* (total counts and spores), and of coliforms in the beds were studied. Bedding samples in the free stalls were collected from the surface and at 10-, 20-, and 30-cm depths, respectively, and from 2 locations at the back, 30 cm from the curb of the stall, and from one place at the front, 150 cm from the curb. Samples were collected on 1 d after replacement, then at 1, 2, 6, and 14 wk, respectively. Samples of bedding at the surface and at 10 cm depth were taken as composite samples from 10 stalls. Samples from 20- and 30-cm depths were taken from 1 randomly selected free stall on each sampling occasion. On each sampling occasion 1 sample from the back part of the free stalls at the surface and at the different depths were analyzed for DM, pH, and a_w , respectively. Two samples of fresh sawdust before use as bedding were analyzed for bacteria and spore counts; 1 sample was analyzed for DM, pH, and a_w .

Preparation of Spores

Spores of *B. cereus* strain A205, originally isolated from the used bedding material (sawdust), were prepared by the surface spreading of 0.1-mL aliquots of a fully grown culture on sporulation agar plates. The sporulation agar had the following composition: 8 g of nutrient broth (Difco, Boule Nordic, Huddinge, Sweden), 1 g of KCl, and 30 g of Bacto-Agar (Difco) per L, with the addition of 10 mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 10 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 10 μM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. The plates were incubated aerobically at 30°C for approximately 1 wk. The proportion of spores was checked regularly by phase-contrast microscopy. The spores were harvested by washing the plates with sterile physiological saline and centrifuging the washings at $7,500 \times g$ for 10 min. They were then washed twice and finally resuspended in saline. The spore suspensions were diluted and frozen at -20°C in aliquots suitable for the laboratory experiments.

Microbiological Analyses

The fresh samples were analyzed for the total counts of *B. cereus* and coliforms. All milk samples of 200 mL

and bedding samples of at least 100 to 200 g were frozen at -20°C until analyses of *B. cereus* spores could be carried out. All samples were analyzed within 3 d. The samples were thawed in cold water just before analysis.

Bedding material weighing 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 Ltd., London, UK) and then homogenized twice in a Colworth stomacher for 30 s each time. Samples with larger particles able to damage a stomacher bag were weighed in the same way into 500-mL sterile glass bottles and shaken on a rotary shaker for 15 min at 300 rpm. From the stomacher bag or glass bottle, 100 mL of liquid was transferred to a sterile 100-mL cylinder. Following 2 min for sedimentation, 1 mL was collected at the 50-mL mark for analyses of vegetative *B. cereus* and coliforms, and 20 mL was transferred to a test tube and heat-treated at 72°C for 5 min for analysis of *B. cereus* spores (Christiansson et al., 1997). Serial 10-fold dilutions were surface-plated in duplicate for the determination of *B. cereus* on blood agar plates [blood agar base No.2 (Oxoid, Basingstoke, UK); 10 mg/kg polymyxin B sulfate (Sigma Chemical, St Louis, MO); and 5% bovine defibrinated blood], and pour plated in duplicate in violet red bile agar (Oxoid) for the determination of coliforms. The blood agar plates were incubated aerobically at 20°C for 48 h, and typical colonies of *B. cereus* with a zone of hemolysis were counted. When necessary, confirmation of identity was made by phase-contrast microscopy and plating on mannitol-egg yolk-phenol red agar (Mossel et al., 1967), and by biochemical typing using API 50 CHB/20E system (bioMérieux, Marcy-àtoile, France). Violet red bile agar plates were incubated at 30°C for 24 h before the colonies were counted.

Milk samples of 100 mL in duplicate were heated in a water bath at 72°C for 5 min and then treated with trypsin and Triton X-100 (Christiansson et al., 1997). The samples were filtered through a membrane filter with 0.8- μm pore size (11404-47-ACN, LKB, Sartorius AB, Sundbyberg, Sweden) using a filtration apparatus equipped with a sterilizable filter support and funnels (Sartorius SM16831). 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 the blood agar plates and incubated aerobically at 20°C for 48 h.

Laboratory Methods

Water activity was determined using a 0.5-g sample in a water activity measurement instrument, the Aqua Lab CX-2 (Decagon Devices, Inc., Pullman, WA) calibrated to distilled water. The determination of DM con-

tent was performed by drying bedding material at 100°C for 24 h and weighing.

Statistical Analyses

Statistical calculations were performed using Minitab version 14 for Windows (Minitab Inc., 2003). Log-transformed values for *B. cereus* (spores and total counts), and coliforms were used for statistical analyses using Pearson correlations and ANOVA. Pairwise comparisons were made by Tukey's test.

RESULTS

Study 1: The Presence of *B. cereus* and Coliforms in Bedding

The samples taken from the sawdust before use as bedding had 45% DM, a_w between 0.95 and 1.00, ≤ 100 *B. cereus* spores/g, ≤ 100 *B. cereus*/g, and < 100 coliforms/g. The temperature measured in 1 free stall varied between 13 and 28°C when the air temperature in the barn was 6.9°C. The highest temperatures (21 to 28°C) were found at the back part of the bed at the 10- and 20-cm depths. Samples of bedding from the free stalls had a DM between 27 and 67%; the lowest DM was found at the 30-cm depth. The a_w was between 0.95 and 1.00 and thus did not limit growth of *B. cereus*.

Bacillus cereus and coliforms occurred in the entire free stall (Figure 1). Eight percent of the total counts of *B. cereus* was found as spores. Correlations were found between the number of *B. cereus* spores and the total counts of *B. cereus* ($r = 0.81$, $P < 0.001$), and between the total counts of *B. cereus* and the coliforms ($r = 0.264$, $P < 0.001$). No correlation could be detected between the *B. cereus* spores and the coliforms.

In the 2 upper layers of the back part of the free stalls (the area most likely to be in contact with udders) on average, $4.1 \pm 0.10 \log_{10}$ *B. cereus* spores, $5.5 \pm 0.10 \log_{10}$ *B. cereus*, and $6.7 \pm 0.10 \log_{10}$ coliforms, were found per gram of bedding.

More spores (0.5 log units), *B. cereus* (0.8 log units), and coliforms (0.8 log units) were found in the back part of the stalls than in the front ($P < 0.001$).

Most spores ($4.6 \pm 0.13 \log_{10}/g$) were found at the 20-cm depth ($P < 0.05$). The total counts for *B. cereus* were found to be more evenly distributed throughout the different depths of the bed, and the only difference noted was between the surface ($4.9 \pm 0.08 \log_{10}/g$) and the most contaminated 20-cm depth ($5.5 \pm 0.12 \log_{10}/g$; $P < 0.001$). Most coliforms were found at the surface and at the 10-cm depth (6.4 ± 0.10 and $6.2 \pm 0.13 \log_{10}/g$, respectively; $P < 0.001$).

The effect of the time after the bedding was given was analyzed for each depth and location in the stalls

(Figure 1). The 2 bottom layers were hard packed and not affected by the daily management, but significant differences between days were found at the surface and at the 10-cm depth. There was active growth of bacteria and the amount of *B. cereus* present increased with the number of days after the bedding had been given. The largest increase observed between d 0 and 4 occurred on the surface at the back part of the stall; an increase of 1.6 log units for total counts and 1.0 log units for spores. A rapid increase of 1.5 to 2.0 log units for the coliforms occurred by d 2 on the surface, both at the back and the front of the stalls, and no further growth could be detected for d 3 or 4. The time after the bedding was added did not affect the coliform counts at the 10-cm depth.

Larger spore content was found in the bulk tank milk during the first sampling period ($2.3 \pm 0.17 \log_{10}$ spores/L) than during the second period ($1.7 \pm 0.14 \log_{10}$ spores/L; $P = 0.027$). The spore content in the bedding material in the 2 upper layers of the back part of the free stalls was not larger during the first sampling period than during the second period.

Study 2: Laboratory Experiments

The results indicated some of the parameters that were of importance for the proliferation of *B. cereus* in the bedding material. Often more than 20% of the total count of *B. cereus* was found as spores in the bedding samples. The amount of spores in proportion to total counts of *B. cereus* in samples varied and no relation was observed to the different conditions of the bedding material during the experiments.

Bedding Materials. Growth of *B. cereus* was inhibited completely in peat, grew considerably in chopped straw, sawdust (in the first experiment), and sand (in the second experiment) (Figure 2). In the first experiment, very little growth occurred in sand, and in the second experiment, not much growth took place in sawdust. Different batches of bedding material were used in the 2 experiments. The pH was 3.8 in the peat, and 7.0, 8.0, and 7.5, respectively, in the sawdust, chopped straw, and sand.

Mixture of peat and sawdust had an inhibitory effect on the growth of *B. cereus* (Figure 3). The best effect was achieved with 50% peat; 25% peat gave some reduction, but no effect was obtained with 10% peat. Mixtures of 10, 25, and 50% peat with chopped straw did not inhibit *B. cereus*. The pH in the sawdust was 7.6 and in the mixtures with 10, 25, and 50% peat was 6.1, 5.0, and 4.5, respectively. The corresponding pH in the mixtures with 10, 25, and 50% peat in chopped straw was 8.3, 6.9, and 4.9, respectively.

Figure 1. Least squares means ($\pm$ SE) counts of A) *Bacillus cereus* spores, B) *B. cereus*, and C) coliforms in sawdust bedding for each free-stall location (front or back) and depth (surface, 10, 20, and 30 cm) on d 0, 2, 3, and 4, respectively, after the addition of fresh bedding. Each mean is based on 3 to 12 samples; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Feces. The extent of bacterial growth was limited by the availability of nutrients. *Bacillus cereus* grew more slowly and less extensively in sawdust with a low content of feces than with larger amounts (Figure 4). No growth of *B. cereus* occurred in sawdust with a content of 1 or 2% feces.

Urine. When urine was the only source of nutrients and no water was added, *B. cereus* grew well in peat ($6.6 \log_{10}/g$) and to a lesser extent in straw ($5.2 \log_{10}/g$) and sawdust ($4.4 \log_{10}/g$), but not in sand ($<3 \log_{10}/g$). The pH in peat was 7.6 and in the other bedding materials was between 9.3 and 9.4.

Figure 2. Growth of *Bacillus cereus* (spores and vegetative) at 30°C in different bedding materials in laboratory experiments (1 and 2). The DM content was 30% except for sand (90%); feces contents 7%; and 3 log₁₀ *B. cereus* spores/g were added to the bedding material at the beginning of the experiments.

DM and a_w . When the DM content of the sawdust was adjusted to between 20 and 45%, there was no inhibitory effect on the growth of *B. cereus*. After an elapsed period of 6 d, however, approximately 6 log₁₀ *B. cereus*/g sawdust was found in all the mixtures. The a_w of the different mixtures were close to 1.00. The desorption and adsorption curves of the DM in sawdust showed identical patterns (Figure 5) and indicated that the DM content must be greater than 70% ($a_w > 0.95$) to inhibit *B. cereus* growth.

Figure 3. Growth of *Bacillus cereus* (spores and vegetative) at 30°C in sawdust and in different mixtures of sawdust or straw with peat. The DM was 30%, the feces content 7%, and 3 log₁₀ *B. cereus* spores/g were added to the bedding material at the beginning of the experiment.

Figure 4. Growth of *Bacillus cereus* (spores and vegetative) at 30°C in sawdust with different feces contents. The DM was 30%, and 3 log₁₀ *B. cereus* spores/g were added to the bedding material at the beginning of the experiment.

Study 3: Bedding Frequency

The sawdust samples taken before use as bedding had <100 *B. cereus* spores/g, ≤100 vegetative *B. cereus*/g, and between <100 and 5.2 log₁₀ coliforms/g.

The effects of the different frequencies of adding fresh bedding material were analyzed for each depth of the bed. There was no significant effect of treatments at

Figure 5. The DM content (wet base) in sawdust as a function of water activity at desorption and adsorption.

Figure 6. Effect of different bedding frequencies (twice a week or daily) on mean ($\pm$ SE) counts of A) *Bacillus cereus* spores, B) *B. cereus*, and C) coliforms in sawdust bedding within each free-stall location (front and back) and depth (surface and 10 cm) immediately after fresh bedding was added (d 0) and before fresh bedding was given (d 3 or 4 and d 1, respectively). Each mean is based on 3 samples; significant differences between bedding frequencies of bedding are indicated as ** $P < 0.01$, and * $P < 0.05$.

the 20-cm depth. Somewhat fewer number of spores and lower total counts of *B. cereus* could be found in different parts of the beds at the surface and at the 10-cm depth before the addition of fresh bedding when daily bedding was used, compared with distribution every 3 or 4 d (Figure 6). No effect of treatment was found for the coliform counts.

Before the addition of fresh bedding, in the area most likely to be in contact with the udders (back part, at the surface, and 10-cm depth), the bedding material had on average fewer *B. cereus* spores (3.8 ± 0.22 log₁₀/g) in the stalls with daily bedding than in the stalls bedded twice a week (4.7 ± 0.18 log₁₀/g; $P = 0.015$).

There were no differences between treatments in the total counts of *B. cereus* or the coliforms.

Study 4: Entire Bed Replacement

The samples of sawdust before use as bedding had 53% DM, a_w 0.998, pH 5.4, <100 *B. cereus* spores/g, <100 *B. cereus*/g, and between 5.6 and 6.4 log₁₀ coliforms/g. The newly replaced beds had a high content of coliforms from the start. After replacing the entire bed, an increase in the total counts of *B. cereus* and spore counts occurred very quickly in all parts and at all depths of the free stalls (Figure 7). After 2 wk, the total counts of *B. cereus* and the spore counts were high in the entire bed, and after 6 wk, had reached the same levels as before replacement. The pattern was the same as that found in study 1; there were more bacteria and spores in the back part than in the front part of the free stall. The DM content in the back part was $51 \pm 6.4\%$, the a_w was 0.998 ± 0.004 , and the pH was 7.0 ± 1.0 .

DISCUSSION

It was observed that *B. cereus* spores, vegetative *B. cereus*, and coliforms were present in the deep sawdust beds in the entire free stall. A large proportion of the total counts of *B. cereus* was found as spores in the beds in the free stall and more so in the laboratory experiments, which showed that a rapid and extensive sporulation occurred after growth. The deep sawdust bedding provided a good environment for growth and spore formation of this bacterium. The spore counts of *B. cereus* in the beds were found to be as high as those found in the soil during the grazing period (Christiansson et al., 1999). On average, 4.1 log₁₀, and up to 6.2 log₁₀, spores/g in individual samples could be determined from the upper layers at the back part of the beds, which would be in contact with the cows' udders. Spores present in the bedding material is the major source of spores in milk from the farm, because vegetative *B. cereus* do not sporulate in milk (O'Donovan, 1959) and the cells are killed by pasteurization. To reduce the risk of spore counts >100 spores/L in the raw milk, the amount of *B. cereus* spores should not exceed 4 log₁₀/g in the bedding material (Magnusson et al., 2007).

The bacteria and spore counts in the bedded stalls varied between a maximum value observed before fresh bedding was given and a minimum value found directly after fresh bedding was added to the free stalls. Bedding once a day instead of twice a week had only a small effect on the maximum *B. cereus* counts reached before the new bedding was added. Daily bedding did not have any effect on the coliform counts in the bedding; the counts were already high after 1 d, as found by Hogan

Figure 7. Effect of entire bed replacement in free stalls on the presence A) *Bacillus cereus* spores, B) *B. cereus*, and C) coliforms in sawdust bedding. Each part of the stalls (front and back) and depths (surface, 10, 20, and 30 cm) were analyzed on different days after full bedding replacement. Each value at the front is based on 1 sample and at the back on 2 samples (geometric means).

and Smith (1997). When fresh bedding was added daily, the bedding material had lower counts of spores and bacteria during a greater proportion of the time than when bedding was added twice a week.

The results of the laboratory experiments agreed with those of Slaghuis et al. (1991), who found higher aerobic spore counts in chopped straw than in sawdust used for bedding. It has been found that sand does not support the growth of coliforms, *Klebsiella*, streptococci, and gram-negative bacteria to the same extent as straw

and sawdust (Fairchild et al., 1982; Hogan et al., 1989). In contrast, Kristula et al. (2005) found high count of *Streptococcus* spp. in sand-bedded free stalls. The present results indicated that there were differences between batches of sand and sawdust, as has been previously observed. Zehner et al. (1986) found differences in bacterial growth between hardwood and softwood sawdust. Bernard et al. (2003) found more *B. cereus* in recycled sand having a larger content of organic matter than in fresh sand.

Peat and mixtures of sawdust and peat inhibited the growth of *B. cereus* in the laboratory experiments; however, peat mixed with chopped straw did not have an inhibiting effect. The inhibitory effect of the peat and the mixtures with sawdust was probably due to the low pH, because *B. cereus* grows best at a pH between 4.9 and 9.3 (Kramer and Gilbert, 1989). The effect of pH with time under barn conditions should be further investigated. In the experiment in which nutrient was added to the different bedding materials, the addition of urine led to an increase in the pH of the peat to 7.6, with *B. cereus* growth as a result. The growth in sawdust and straw was less when urine was added and pH was 9.3 or above. Alternative bedding materials such as sand and peat could provide less favorable conditions for the proliferation of *B. cereus*. Different bedding material might have different propensities for attaching to the teats and thus affect the possibility of contaminating the milk (Zdanowicz et al., 2004).

No growth of *B. cereus* occurred with small amounts (1 to 2%) of feces added to the sawdust. These observations were in agreement with those of Zdanowicz et al. (2004) who found a correlation between the cleanliness of the free stalls and the bacterial counts in the bedding. Clean alleys and well-designed free stalls would be of importance in reducing the amount of feces remaining in the stalls.

Zdanowicz et al. (2004) found that the DM content of the sawdust bedding material was correlated to the bacterial counts in the bedding; kiln-dried sawdust had been used and the lowest DM found was 71.7%. In the present laboratory study with DM contents between 20 and 45%, no inhibitory effect on bacterial growth could be detected. It is known that the a_w should be <0.95 to inhibit the growth of *B. cereus* (Kramer and Gilbert, 1989). According to the desorption and adsorption of sawdust in this study, the DM in sawdust must be greater than 70% to achieve an a_w less than 0.95. During the conditions of the present studies when sawdust before use had a DM of 45 to 53%, it would not be possible to keep the DM in the beds at that level.

Completely removing the bedding material and filling the free stalls with fresh sawdust only temporarily reduced the bacteria and spore counts of *B. cereus* in the

beds for 1 to 2 mo. It is not a practical option to replace the beds that frequently. The beds were replaced in a limited number of the free stalls and the *B. cereus* occurring in high numbers in the surrounding environment was easily introduced to the new beds. The high numbers of *B. cereus* found in the deep layer of the beds indicated that contamination could also have occurred from below. The effect of bed replacement on the coliforms was insignificant because the fresh sawdust in this experiment contained very high counts of coliforms at the beginning of the study.

Even when high spore counts occurred in the beds, acceptable milk quality (<100 *B. cereus* spores/L) was achieved. High spore contents were found in the bulk tank milk during the first period in study 1, but a more effective teat-cleaning method was used during the second period, probably contributing to the observed decrease in the milk spore contents of this period (Magnusson et al., 2006). The free stalls were also well managed: bedded twice a week with a large amount of sawdust (75 L/stall) and cleaned thrice a day.

In conclusion, high numbers of *B. cereus* spores can be found in deep sawdust-bedded free stalls. The possibility of permanently reducing the spore levels in the beds through the management of the free stalls appeared to be limited, because there is a continuous growth of *B. cereus*. Free-stall management can, to some extent, reduce the content of spores by frequent bedding and cleaning to prevent the feces and urine from supplying nourishment for bacterial growth. In housing systems with deep sawdust-bedded free stalls, good management of stalls, clean alleys, and effective teat-cleaning are of importance to maintain good milk quality.

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