

***Bacillus cereus* in the
Housing Environment of Dairy Cows**

**Contamination Routes, Effect of Teat-Cleaning, and
Measures to Improve Hygiene in the Cubicles and Alleys**

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Abstract

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The spore-forming bacterium *Bacillus cereus* can survive pasteurisation and thus a limiting factor for the shelf life of pasteurized milk. The occurrence of *B. cereus* spores in the housing environment of dairy cows was investigated and the routes of contamination elucidated also measures to improve hygiene in cubicles and alleys were tested.

High numbers of spores were found in deep sawdust bedded cubicles hence a source of contamination of milk via soiled teats. In laboratory tests, type of different bedding material, pH, and the availability of nutrient in form of faeces in the beds were shown to be important factors for the growth of *B. cereus*. In deep sawdust bedded cubicles more frequent adding of fresh bedding and entire bed replacement had a limited effect on reducing spore content in the beds.

It was shown by experimentally feeding spores to cows that highly contaminated feed could be a potential source of contamination via faeces and soiled teats.

The effects of different premilking teat-cleaning methods were evaluated on experimentally spore contaminated teats. The most effective method in reducing the milk spore content (96 % reduction) was the use of a moist washable towel with or without soap followed by drying with a dry paper towel.

Mechanical scrapers on top of the slatted floor reduced the amount of manure on alley floor, hence reducing the faecal contamination in the cubicles, and improving the hygiene score of udder and teats. The drainage capacity of slatted floors was tested in a laboratory arrangement. For loose faeces from high-yielding dairy cows, the greater void ratio the better drainage capacity, irrespective of slat and slot widths was obtained.

Keywords: *Bacillus cereus* spore, bedding material, cleanliness, contamination, dairy cattle, milk quality, slatted floor, scraper, teat cleaning, udder hygiene.

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Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

- I. Magnusson, M., Christiansson, A. & Svensson, B. 2007 *Bacillus cereus* spores during housing of dairy cows: Factors affecting contamination of raw milk. *Accepted for publication in Journal of Dairy Science* doi:10.3168/jds.2006-754.
- II. Magnusson, M., Svensson, B., Kolstrup, C. & Christiansson, A. 2007. *Bacillus cereus* in free stall bedding. Submitted.
- III. Magnusson, M., Christiansson, A., Svensson, B. & Kolstrup, C. 2006. Effect of different premilking manual teat-cleaning methods on bacterial spores in milk. *Journal of Dairy Science* 89:3866-3875.
- IV. Magnusson, M., Herlin, A.H. & Ventorp, M. 2007. Scrapers on slatted floors for dairy cows: Effect on hygiene. Manuscript.
- V. Magnusson, M., Ventorp, M. & Nilsson, C. 2007. Drainage capacity of concrete slatted floors for dairy cattle. Submitted.

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My own contribution to the papers included in this thesis has been as follows:

- I: Took part in planning and data evaluation. Performed sampling and data collection apart from laboratory work. Responsible for writing the paper.
- II: Took part in planning and data evaluation. Performed major part of sampling and data collection apart from laboratory work. Responsible for writing the paper.
- III: Planning of experiments in collaboration with co-authors. Performed sampling and data collection apart from laboratory work. Responsible for analyzing the data and for writing the paper.
- IV: Planning of experiment in collaboration with co-authors. Responsible for sampling and data collection, analyzing the data, and for writing the paper.
- V: Planning of experiment in collaboration with co-authors. Responsible for the data collection, analyzing the data, and for writing the paper.

List of abbreviations

cfu	colony forming unit
DM	dry matter
RAPD-PCR	random amplified polymorphic DNA polymeras chain reaction
RCA	reinforced clostridial agar
SCC	somatic cell count
UHT	ultra high temperature
VRA	violet red bile agar

Background

The housing of dairy cattle is an important means of keeping them accessible to the farmers under practical conditions, in order to breed, feed, handle and milk them. Dairy housing is a compromise between different demands. It should provide good animal welfare and health, while at the same time appropriate conditions for high quality milk production, an efficient and good working environment for the operators, suitable facilities for the storage and handling of milk, feed, bedding and manure, and all within the economic limits of being profitable. This housing is not very clean and is not an optimal place to produce foodstuffs, but with good planning, appropriate working equipment and good management, the vast majority of the Swedish farms manage successfully to produce high quality milk.

In order to manufacture a variety of dairy products, the quality of raw milk has to fulfil certain hygienic criteria. General important factors for the microbiological quality of milk are: healthy cows without mastitis, high quality feed - especially good quality silage without *Clostridium* spores, clean animals – especially the udder and teats, good milking routines and hygienic conditions during milking, and a clean, well-managed and maintained milking equipment.

The presence of the spore forming bacterium *Bacillus cereus* in pasteurised milk limits its shelf life (Griffiths, 1992). *B. cereus* is commonly found in the soil. Inferior keeping quality caused by high levels of *B. cereus* spores in raw milk is most prominent during the summer and autumn when cows are at pasture. Periodically, the problem can occur during indoor confinement, and on some farms high levels of *B. cereus* spores can be found in raw milk. According to information from the Swedish dairy industry (Pers. information), high spore levels during the housing period has to some extent been associated with farms with cubicles or other loose housing systems. No systematic investigation has been undertaken to evaluate the relative importance of different contamination sources of *B. cereus* in milk under contemporary housing conditions. More and more farms in Sweden change their housing system from tie-stalls to systems with cubicles. It is important to evaluate if these or similar systems may have negative side-effects with respect to *B. cereus* and milk quality.

Introduction

Bacillus cereus

Bacillus cereus is a commonly occurring soil saprophyte and is easily spread in the environment, e.g., to plant material, air, dust and water (Labots *et al.*, 1965; Kramer & Gilbert, 1989; Slaighus *et al.*, 1997). It may also be isolated from different processed foods, such as rice, meat, spices, eggs and dairy products (Kramer & Gilbert, 1989).

B. cereus is a gram-positive, aerobic or facultatively anaerobic spore-forming, motile, rod-shaped bacterium. The optimal growth temperature is 28-35°C, but growth typically occurs between 10-50°C (Kramer & Gilbert, 1989). Psychrotrophic strains able to grow at as low as 5°C have been identified (Griffiths & Phillips, 1990). *B. cereus* can be divided, on basis of growth temperature, into psychrotrophic and mesophilic strains. Psychrotrophic strains grow at 5°C and relative rapidly at 10°C. Mesophilic strains fail to grow below 8°C and only grow slowly at 10°C (Sutherland *et al.*, 1996). *B. cereus* multiply in a pH range of 4.9 – 9.3 and the minimum water activity for growth is 0.91-0.95 (Kramer & Gilbert, 1989).

Under stress conditions, such as nutrient exhaustion, the *B. cereus* cells sporulate. The spores can then convert back to a vegetative cell (germinate). Factors affecting growth and germination are, e.g., temperature, pH, and oxygen level, and the presence of certain nitrogen and carbon compounds (Vlaemynck & Van Heddeghem, 1992). The spores of *B. cereus* are very adhesive to different surfaces, especially those that are hydrophobic. This adhesion is mainly due to the spore surface hydrophobicity and the spores being covered with long appendages (Rönner *et al.*, 1990). *B. cereus* spores are thermally resistant and can survive milk pasteurisation. The psychrotrophic spores will thus germinate and grow during storage at refrigeration temperatures (Kramer & Gilbert, 1989). Pasteurisation will actually induce spore germination, and in the absence of competing flora, *B. cereus* grows well (Granum & Lund, 1997).

Problems caused by *B. cereus*

Limited shelf life of milk

Psychrotrophic strains of *B. cereus* are some of the most important organisms limiting the shelf life of pasteurised milk stored above 6°C (Griffiths, 1992, Ternström *et al.*, 1993). The bacterium may cause the aggregation of the creamy layer of pasteurised milk, known as bitty cream (Billing & Cuthbert, 1958), which can be explained by the lecithinase activity of *B. cereus*. *B. cereus* is also responsible for sweet curdling, without pH reduction, both in homogenized and non-homogenized pasteurised milk (Overcast & Atmaram, 1974). The concentration of psychrotrophic *B. cereus* in pasteurised milk is higher in the summer than in winter time (Phillip & Griffiths 1986, Larsen & Jørgensen, 1997).

Mesophilic strains do not have any effect on the shelf life of refrigerated milk, since they do not grow at low temperatures, but they can serve as a breeding ground for the colonisation of other bacteria in biofilms, if they are allowed to colonize the dairy equipment (Kumar & Anand, 1998).

Food poisoning

B. cereus is also a potential food poisoning organism that can produce three or more enterotoxins and one emetic toxin causing diarrhoea and vomiting, respectively, in humans (Granum & Lund, 1997; Kramer & Gilbert, 1989). For both the diarrhoeal type and the emetic type of poisoning, the food involved has usually been heat treated, and spores surviving that then germinate and grow cause the food poisoning. Concentrations of viable *B. cereus* in food ranging from 10^3 to 10^{10} cfu/ml have been implicated in food borne disease outbreaks (Kramer & Gilbert, 1989; Andersson *et al.*, 1995). Both the psychrotrophic and the mesophilic strains have been shown to produce toxins. The mesophilic *B. cereus* are of importance as contaminants when milk powder is produced from the milk (Becker *et al.*, 1994). Both the diarrhoeal strains (In't Veld *et al.*, 2001), and the emetic toxin producing strains of *B. cereus* (Svensson *et al.*, 2006) have been found in milk.

Restrictions

In Sweden milk has to be pasteurised before sale. The legislation was enacted already in 1937 to prevent the transmission of tuberculosis. Today the aim is to kill pathogenic bacteria such as, *Salmonella*, *Listeria*, *Campylobacter* and Enterohemorrhagic *Escherichia coli*.

The pasteurised milk in Sweden is stored at $\leq 8^\circ\text{C}$. The shelf life (best before-date) is normally 6 days during the summer time and 7 days during the winter time. The growth of *B. cereus* is generally the limiting factor for milk shelf-life during the grazing period. According to the Swedish food act (1971) valid until 2006, no more than 10^3 cfu/ml had been allowed in pasteurised milk on the best before-day. At a level of 10^4 cfu/ml it had been then illegal to sell the milk. There are no microbiological criteria for *B. cereus* in the new EC food hygiene legislation valid from 2006 (European Union, 2005), but *B. cereus* amounts above 10^3 - 10^5 /g in food is considered to be potentially harmful (EFSA, 2005). Therefore the Swedish National Food Administration still considers more than 10^4 cfu/ml to be unsafe (Livsmedelsverket, 2006). To fulfil these requirements, there must be less than 10^2 spores/l milk at the packing stage. The dairy industry includes *B. cereus* in the milk grading system and payment scheme to the farmers to assure that the raw milk has a good quality. The farmers are paid less for delivered milk that contains more than 200 *B. cereus* spores/l (Arla Foods, 2006).

Handling of milk at the dairy plant

The pasteurisation of milk is carried out at a high temperature for a short time (at least 71.7°C for 15 s, or any equivalent combination). Immediately after pasteurisation, the milk must be cooled to 6°C or below. Pasteurised milk has a shelf life from only a couple of days in some countries to up to over 20 days in, e.g., USA (Rysstad & Kolstad, 2006). The difference in keeping quality is dependent on local legislation, raw milk quality, processing method, hygiene during the filling of the containers and the temperature of the cold chain (maintenance of refrigeration from processing to the store). The very long shelf life of pasteurised milk in the USA can largely be attributed to a very good cold chain. For every 2°C increase in the storage temperature, the shelf life is reduced by 50% (Rysstad & Kolstad, 2006).

UHT-milk is heat treated to at least 135°C for 1 s or more; the product is then packed aseptically and can be distributed in ambient temperature. At this heat treatment temperature the *B. cereus* spores will be killed. The shelf-life is thus dependent on heating temperature, and time and type of heat treatment (Rysstad & Kolstad, 2006). However, the products do not have the same fresh taste as pasteurised milk.

To remove spores from the milk, bactofugation can be used, which removes bacteria and especially spores, from the milk by high centrifugation. This process can remove 94 - 98% of the aerobic spores. Microfiltration is another method, and is even more efficient in removing bacterial cells and spores from milk since it removes between 99.1 and 99.9% of the aerobic spores (te Giffel & Van der Horst, 2003). The use of bactofugation or microfiltration in addition to heat treatment is a matter of cost. In cheese processing, bactofugation or microfiltration is commonly used in combination with a heat treatment for removal of the *Clostridium* spores to prevent late blowing in some types of cheeses. It is likely that the removal of spores from raw milk by microfiltration may be a solution in the future that could be used in fluid milk processing in combination with pasteurisation (Barbano *et al.*, 2006)

Recontamination of the milk can occur at the dairy plant (te Giffel *et al.*, 1996; Lin *et al.*, 1998, Svensson *et al.*, 1999). Problems may occur along the whole processing line. Svensson *et al.* (2004) found problems in the silo tanks. A pasteurizer (Svensson *et al.*, 2000), and the filling machines (Eneroth *et al.*, 2001) have also been shown to be possible sources of *B. cereus* contamination. However, the results of several studies indicate that the major contamination of *B. cereus* comes from the farm raw milk (Crielly *et al.*, 1994; Lin *et al.*, 1998; Griffith & Phillips, 1990; Svensson *et al.*, 1999).

Contamination of raw milk

B. cereus spores from the farm environment can easily find their way into the raw milk. Possible sources of contamination are the soil, faeces, bedding, feed, air, the milker, and the milking equipment (Van Heddeghem & Vlaemyneck, 1992). More

bacteria and spores are found during the summer and autumn than during the winter. The seasonal variation of vegetative *B. cereus* in pasteurised milk (Phillip & Griffiths, 1986; Larsen & Jørgensen, 1997) is similar to the seasonal variation of the psychrotrophic numbers of spores found in the raw milk (McKinnon & Pettipher, 1983; Phillip & Griffith, 1986; Crielly *et al.*, 1994).

Cows on pasture are the reason for the seasonal variation of *B. cereus* in raw milk (Slaghuis *et al.*, 1997), since there is a risk of the teats being contaminated by the soil. Bad weather or rainy days can affect the milk spore content. The high water content of the soil and dirty access alleys have been shown to be important factors correlating with a high number of spores in milk (Christiansson *et al.*, 1999).

B. cereus spores in milk can also be found during the housing period of dairy cows (Slaghuis *et al.*, 1997). The udder and teats are not in contact with the soil as they are during the grazing period, but the teats can be contaminated with faeces and bedding material. Aerobic spores and spores of *B. cereus* have been found in feed and faeces (Torp *et al.*, 2001; te Giffel *et al.*, 2002), and in used bedding material (McKinnon & Pettipher, 1983, Slaghuis *et al.*, 1991; Crielly *et al.*, 1994). Air has been suggested to be a source of contamination (Stewart, 1975), as well as the milking equipment (Labots *et al.*, 1965). However, the importance of the different sources and contamination routes are not completely clear.

Structure of the thesis

In the present thesis several aspects of the possible contamination routes of *B. cereus* have been investigated (Figure 1). Four main areas are discussed: the sources and contamination routes of *B. cereus* during the housing period (Paper I); the possibility of reducing the presence of *B. cereus* in the bedding material (Paper II); the effect of premilking teat-cleaning methods on bacterial spores in milk (Paper III); and the possibility of improving the hygiene of the floors in the alleys and the effect on the cleanliness of the cubicles and teats (Papers IV and V).

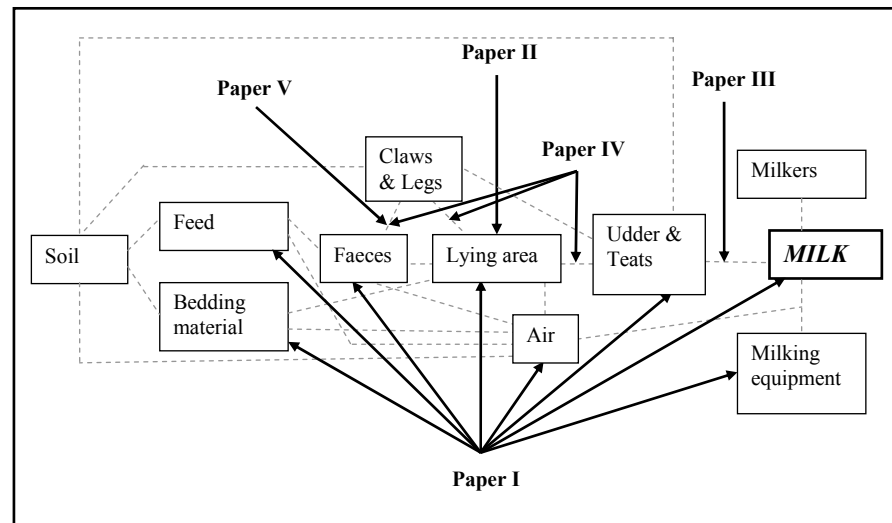


Figure 1. Possible contamination routes of *Bacillus cereus* related to an overview of the areas that have been studied in Papers I – V.

Aims

The aim of this thesis was to identify the source and routes of contamination of *B. cereus* in raw milk during the housing period of dairy cows. Furthermore, the ambition was to find measures which could be taken in the housing environment of the dairy cows and management procedures which could improve the udder and teat cleanliness, and reduce the presence of spore contamination in milk.

The specific objectives were to:

- Identify contamination sources and routes of contamination with *B. cereus* spores during the housing period of dairy cows (Paper I).
- Investigate measures to reduce the presence of *B. cereus* in the bedding:
 - Investigate factors of importance to the growth of *B. cereus* in bedding material (Paper II).
 - Determine the possibility of reducing the presence of *B. cereus* in deep sawdust bedded cubicles by using different management routines, such as the daily addition of bedding or the entire replacement of the beds (Paper II).
- Investigate the effect of using different premilking teat-cleaning methods on the presence of bacterial spores in raw milk (Paper III).
- Investigate measures to improve the cleanliness of cubicles, and the udder and teats:
 - The effect of alley floor cleanliness on the faecal contamination of cubicles and the cleanliness of the udder and teats (Paper IV).
 - The effect of scrapers on the slatted floor on the manure accumulation in the alleys (Paper IV).
 - The effect of slatted floor design on the drainage capacity (Paper V).

Materials and methods

The research included in this thesis is based on several studies presented in five papers (Table 1). All studies on farms were carried out in the southern part of Sweden during the housing period, August – April. The details of the experimental procedures, sampling and data collection, and analyses are described in Papers I - V.

Sources and contamination routes of *B. cereus* (Paper I)

Four studies were conducted in order to identify the contamination sources and the contamination routes of *B. cereus* in dairy cow housing environment.

The first study was carried out over a two week period at the Alnarp Dairy Research Station, Swedish University of Agricultural Sciences, which had two different cow housing systems (tie-stalls and cubicle housing). The occurrence of *B. cereus* spores in the feed, air, faeces, fresh and used bedding material, and rinse water from the milking equipment was compared with the spore level in the bulk tank milk on ten sampling occasions. The cleanliness of all cubicles and of 10% randomly selected cows' udder and teats was observed and recorded.

A study similar to that at the Research Station was conducted on a farm previously observed to have elevated spore contents in the bulk tank milk (cubicle housing with 30 cm deep sawdust bedded cubicles). In addition, 125 isolates of *B. cereus* from the different sources (used bedding, air, rinse water, and bulk tank milk) were collected on this farm during a three month period and compared by RAPD-PCR fingerprinting to establish identity between the isolates and thereby the routes of contamination.

In order to confirm or reject results found in the two previous studies, a less extensive study was carried out for two weeks on an additional five farms. Two farms having 5-20 cm deep sawdust bedded cubicles, one farm having deep sand bedded cubicles, one farm having a deep straw-bed, and one farm having tie-stalls were studied. Samples were taken on four to seven sampling occasions from the used bedding material, rinse water and bulk tank milk.

The contamination route of *B. cereus* from feed to faeces to milk was studied in a controlled feeding experiment where the cows were experimentally fed *B. cereus* spores for seven days. The cows were kept in a separate building at Alnarp Dairy Research Station. Twelve cows in tie-stalls were assigned to four groups of three cows in each group. The control group did not receive any spores and the three other groups were fed spores, with the purpose of achieving about 10^4 , 10^5 , and 10^6 spores/g faeces, respectively. Samples of milk and faeces from each cow, and air samples from each group were collected during the last three days of the spore-feeding period. Visual inspection and scoring were made of the cleanliness of the udder and the teats.

***B. cereus* in deep bedded cubicles (Paper II)**

To investigate the possibility of reducing the presence of *B. cereus* in deep bedded cubicles the following studies were carried out; partly as a laboratory test at the Swedish Dairy Association, Research and Development Department, Lund, Sweden, and partly at the farm previously observed to have elevated numbers of *B. cereus* spores in bulk tank milk and in the bedding material (Paper I). The farm had about 300 Swedish Holstein Frisian cows in cubicles with 30 cm deep sawdust beds. The cubicles were bedded twice a week with 75 l of sawdust per cubicle.

The occurrence of *B. cereus* (total counts and spores), and coliforms in the deep bedded cubicles was investigated at the farm during two 14-day periods. Samples were collected on 13 sampling days from the front and the back part of the cubicles, and at four different depths of the beds. Sampling took place immediately after the fresh bedding was added to the cubicles and then after two, three, and four days. Some of the bedding samples were analysed for DM, and water activity.

Laboratory studies were carried out to study factors influencing the growth of *B. cereus* in the bedding material. The standard condition was set to reproduce natural conditions in bedding in the cubicles. The incubation temperature was 30°C, 7 % DM faeces was added to the bedding material, and the DM content was 30% in all the bedding material except for sand, which had a DM of 90%. *B. cereus* spores were added to the bedding material and the bacterial growth of *B. cereus* was studied for 6 to 8 days. The pH of the bedding material was also determined. The factors studied under these controlled condition were the different types of bedding material (sawdust, sand, straw, and peat), the addition of urine instead of faeces to the bedding, different percentages of faeces added to sawdust (1-10%), and different DM of sawdust (20-45%). The relationship between the DM and water activity in sawdust was evaluated.

The effect of the frequency of adding new bedding material to the cubicles was studied in a group of 140 lactating dairy cows. Fourteen cubicles in one row in the centre of the barn were bedded with 75 l sawdust per cubicle twice a week, and the opposite row with 14 cubicles was bedded daily with 30 l of sawdust/cubicle. Samples of sawdust in the beds were collected on six occasions during a 14-day period. The bacterial growth of *B. cereus* (total counts and spores), and coliforms, was studied in the front and the back part of the cubicles and at three different depths of the bed.

The effect of total bed replacement was studied in a group of 140 lactating cows. Fourteen cubicles in one row in the centre of the barn was completely cleaned, all the old bedding was removed and replaced with fresh sawdust. The bacterial growth of *B. cereus* (total counts and spores), and coliforms in the newly replaced beds was studied at five occasions during the first 14 weeks after bed replacement. Samples of bedding material were collected at the back and the front part of the cubicles and at four different depths of the beds. Some of the samples were analysed for pH, DM, and water activity.

Teat-cleaning and bacterial spores in milk (Paper III)

The effect of different premilking teat-cleaning methods on the presence of bacterial spores in milk was evaluated in three controlled experiments at Alnarp Dairy Research Station.

Twelve teat-cleaning methods using dry or moistened towels and different combinations of methods using soap or two types of towels, together with 10 or 20 s cleaning time were compared to a control (no cleaning, no forestripping) in two Latin-square designed experiments with seven cows, seven treatments and four replications (two milkers and morning vs. evening milking) in each experiment (Paper III, Table 1 and 2). The teats were contaminated with a faeces-water slurry containing a large number of *Cl. tyrobutyricum* spores.

The best cleaning method was evaluated in an additional experiment to determine the effect of different teat contamination mixtures. The effects of different contamination carriers (faeces, sawdust, and soil), different type of spores (*B. cereus* and *Cl. tyrobutyricum*), and the degree of contamination (faeces and extra faeces) were studied in a Latin-square designed experiment with eight cows and eight treatments and two replicates (morning vs. evening milking; Paper III, Table 3).

Milk yields were recorded and milk samples were collected from each cow at each milking occasion and analysed for bacterial spores. To determine if natural contamination with spores occurred during the experiment, samples were taken from the milking equipment, faeces, and bedding material, and the cleanliness of the teats were recorded.

Alley-flooring and hygiene (Paper IV and V)

The effect of electronically controlled mechanical scrapers on slatted floors on the accumulation of manure on the alley floor and the consequences for the faecal contamination of cubicles and the cleanliness of the udder and teats were studied at Alnarp Dairy Research Station. The floor design of the slatted floor affects the drainage capacity and thereby the amount of manure accumulating in the alleys. The drainage capacity of concrete slatted floors with different slats and slot widths was studied in a laboratory test.

The effect of the scrapers on slatted floors was studied in two groups of 21 cows each on a slatted rubber floor, with and without scrapers, respectively. The cleanliness of the alleys, cubicles, and the udder and teats were studied once weekly for a three week period. Manure was collected from 10 different samplings places for each group and on each occasion to determine the manure accumulation on the alley floor. Bedding samples were collected from the back part of nine cubicles per group for the determination of faecal contamination. The cleanliness of the udder and teats of all the cows was visually observed and scored at the morning milking (Paper IV).

The drainage capacities of concrete slatted floors were tested in a laboratory arrangement (Svennerstedt, 1999), using faeces of two consistencies, obtained from high-yielding dairy cows and from dry cows. Faeces and water (simulating urine) were dropped on the floors, which were trampled by a dummy with claws similar to those of a cow. The amounts of faeces and water draining through the slats were recorded. The test was performed with four replicates on each tested floor setup and faecal consistency. Twenty-two different floor arrangements with different combinations of single slats ranging from 50 to 150 mm, together with slots from 20 to 50 mm were tested (Paper V, Table 1). The void ratio (the ratio between the slot area and the total floor area) of the floors varied between 16.7 and 33.3 %.

Summary of sampling and data collection

Sampling procedures

For microbiological analyses, the milk samples were collected from bulk tank milk (Paper I and II). Individual milk samples were collected with Tru-test milk meters (Papers I and III). Samples of rinse water from the milking equipment were collected before milking at the outlet and at the releaser after the circulation of water (Papers I and III), or as residual rinse water, remaining in the milking equipment from the last cleaning cycle (Paper I).

Air samples were collected for 30 s at udder height close to the animal during milking with a RCS-sampler (Biotest Diagnostics, Soest, Netherlands) equipped with blood agar strips (Paper I).

Sample of feed (Paper I) and fresh bedding material (Papers I, II, and III) were collected and analysed. The bedding material was collected from the back part of cubicles/tie-stalls as composite samples (Paper I). In deep sawdust bedded cubicles the bedding material was collected from one cubicle on each sampling day at different depths (0, 10, 20, and 30 cm; Paper I). When the presence of *B. cereus* spores in the beds was more closely investigated samples were collected at the surface and at 10 cm depth as composite samples on each sampling occasion from ten cubicles. Samples from the 20 and 30 cm depths were collected from one cubicle on each sampling occasion (Paper II). Faeces were collected as composite samples (Papers I and III), and as individual samples in the feeding experiment (Paper I). All fluid and solid samples were frozen at -20°C, until analysed (Papers I, II, and III).

To determine the amount of manure accumulating on the slatted floors, the manure in the alleys found within a frame of 500 x 1000 mm was collected and the samples were weighed (Paper IV). The faecal contamination of the cubicles in Paper IV was determined by analysis of the ash content in bedding samples taken from an area of 600 x 600 mm in the back part of the cubicles. The amount of faeces present was calculated from the ash content in each sample. The site and size of the area was assumed to reflect the area where the udder normally came in

contact with the cubicle floor. Samples were collected in the morning before the cubicles were cleaned.

Hygiene scores

All visual observations were made by the same person. The dirtiness of the udder, teats, and teat tips of the cows were scored before the morning milking on a 5-point scale (Christiansson et al, 1999) where: 1 = completely clean; 2 = some visual dirt; 3 = less than 25% of the area dirty; 4 = 25 - 50% of the area dirty; and 5 = more than 50% of the area dirty (Papers **I** and **III**). In Paper **IV**, the scoring was modified and the dirtiness of the udder, and teats of all cows was linear scored between 0 and 100 by marking on a 100 mm line, where 0 = completely clean, and 100 = completely covered with dirt.

The cleanliness of all cubicles was observed in the morning before they were cleaned. Cubicles were recorded as being contaminated if a minimum area of 100 cm² was covered with faeces, and the number of contaminated cubicles was noted (Paper **I**).

Summary of analyses and microbiological methods

Preparation of spores

The experimental addition of *B. cereus* spores to the feed was used in the feeding experiment (Paper **I**), and in the laboratory experiment when different growth factors of *B. cereus* in the bedding material were studied (Paper **II**). In the teat-cleaning experiment, both *B. cereus* spores and *Cl. tyrobutyricum* spores were used (Paper **III**). The cultivation and preparation of spores were carried out at the Swedish Dairy Association, Research and Development Department, Lund, Sweden.

Microbiological analyses

The analyses of all fluid and air samples for spores of *B. cereus*, and solid samples for vegetative *B. cereus* and coliforms were carried out at the Swedish Dairy Association, Research and Development Department, Lund, Sweden. Analyses of solid samples for spores were carried out at the Department of Agricultural Biosystem and Technology, SLU, Alnarp, Sweden.

The milk and water samples were analysed for the presence of *B. cereus* (Papers **I**, **II** and **III**), and *Cl. tyrobutyricum* spores (Paper **III**). The samples were heat treated to kill vegetative bacteria using a water bath at 72°C for 5 min, and analysed by filtration (Christiansson *et al.*, 1997). Filters for evaluating *B. cereus* counts were placed on the surface of blood agar plates and incubated aerobically at 20°C for 48 h. For evaluating *Cl. tyrobutyricum* counts, the filters were placed on RCA agar plates with D-cycloserine and incubated anaerobically at 37°C for 72 h.

Blood agar strips from the air sampler were incubated aerobically at 20°C for 48 h before counting of *B. cereus* colonies (Paper I).

Solid samples (feed, faeces, bedding material) were heat treated at 72°C for 5 min to kill the vegetative bacteria before analyzing for the presence of *B. cereus* spores (Papes I, II and III), and at 80°C for 10 min before the analysis for the presence of *Cl. tyrobutyricum* spores (Paper III). The samples were analysed for *B. cereus* counts on blood agar plates incubated aerobically at 20°C for 48 h, and for *Cl. tyrobutyricum* counts on RCA agar plates with D-cycloserine and incubated anaerobically at 37°C for 72 h. The bedding material was analysed for total counts of *B. cereus* on blood agar plates incubated at 20°C for 48 h, and for coliforms on VRA plates incubated at 30°C for 24 h (Paper II).

Fingerprints with RAPD-PCR

Analyses of RAPD-PCR fingerprints were carried out at the Swedish Dairy Association, Research and Development Department, Lund, Sweden. *B. cereus* colonies were collected randomly from each of the filters and plates. A DNA template for PCR-amplification was extracted from pure cultures according to the method described by Nilsson *et al.* (1998). RAPD-PCR analysis was performed using primer (5'CCGAGTCCA 3'; Pharmacia Sweden) and AmpliTaq DNA-polymerase (Perkin Elmer, USA). The PCR products were separated on an agarose gel, visualized on an UV transilluminator, and photographed and scanned. The evaluation of RAPD-patterns was strictly standardized and enabled data from different sampling occasions to be combined in dendrograms. (Paper I)

Laboratory methods

The water activity of sawdust was determined on 0.5 g samples in an Aqua Lab CX-2 (Decagon Devices, Inc. USA) calibrated with distilled water (Paper II). DM analyses were performed on samples by drying in a drying cabinet at 100°C for 24 h (Papers II, IV, and V). The ash content was determined after the samples had dried at 100°C for 24 h. The samples were ground through a 1 mm sieve and duplicates of two grams of each sample was put in an oven at 650°C for 30 min (Paper IV). The consistency of faeces was measured by the determination of DM and the slump height (Paper V).

Statistical analyses

Statistical calculations were performed using MINITAB version 14 (Minitab, 2003), and SYSTAT version 9.0 (SPSS, 1999). The statistical methods have been described in detail in each paper. Log-transformed values for bacterial and spore counts, and manure accumulation on alley floors, and observed values for other parameters were generally used for the statistical analyses by the Pearson correlation and ANOVA procedures (Papers I, II, III and IV). In cases when log-transforming did not show a normal distribution, non-parametric analyses such as the Wilcoxon signed rank test and MannWhitney U-test were used (Paper I). The RAPD-PCR results were analysed using Fisher's exact test (Paper I). The

relationship between drainage capacity and the different properties of concrete slatted floors were analysed by the multiple linear regression procedure (Paper V).

Table 1. *An overview of the studies according to paper*

Paper	General area studied	Treatment	Main parameters
I	2 different housing systems	Tie-stall, cubicle housing	<i>B. cereus</i> in: feed, faeces, air, bedding material, rinse water, milk. Cleanliness of: cubicles, udder and teats.
	Farm with elevated counts of <i>B. cereus</i> in milk	Cubicle housing with deep sawdust bedded cubicles	<i>B. cereus</i> in: feed, water cups, faeces, air, bedding material, rinse water, milk. Cleanliness of: cubicles, udder and teats.
	5 farms with different types of housing systems	Deep sawdust bedded cubicles (2), Deep sand bedded cubicles, deep straw-bed, and tie-stall	<i>B. cereus</i> in: bedding material, rinse water, milk.
	Spores in milk - feeding experiment	Cows fed spores, 4 x 3 cows	<i>B. cereus</i> in: faeces, air, milk.
II	Deep sawdust bedded cubicles	Survey	<i>B. cereus</i> coliforms
	Bedding material - laboratory study	Different bedding materials, DM, water activity, faeces as nutriment	<i>B. cereus</i>
	Deep sawdust bedded cubicles	Frequency of adding fresh bedding material, daily or twice a week	<i>B. cereus</i> coliforms
	Deep sawdust bedded cubicles	Total bed replacement	<i>B. cereus</i> coliforms
III	Spores in milk	6 different premilking teat-cleaning methods and control	<i>Cl. tyrobutyricum</i>
	Spores in milk	6 different premilking teat-cleaning methods and control	<i>Cl. tyrobutyricum</i>
	Spores in milk	Different contamination matrices (faeces, sawdust, soil), type of spores, and degree of contamination	<i>B. cereus</i> <i>Cl. tyrobutyricum</i>
IV	Slatted floor in alleys	With or without scrapers	Manure on alley floor Faeces in cubicles Cleanliness of udder and teats
V	Slatted floor - laboratory study	Different slat and slot widths	Drainage capacity

Results

Sources and contamination routes of *B. cereus* (Paper I)

The numbers of *B. cereus* spores found in samples from the two housing systems at the Research Station were low, often below the detection level, both in the milk and in samples from the feed, air, bedding, faeces and rinse water. There were no differences between the two housing systems.

The farm having a previously observed elevated number of spores in milk had on average significantly more *B. cereus* spores than those found at the Research Station in the milk (81 spores/l), rinse water at two sampling points (60 and 194 spores/l, respectively), air (275 spores/m³) and in the used bedding material (17,800 spores/g). A high amount of spores was also found in the water remaining in the water cups (> 1500/l). Low amounts of spores (< 50 spores/g) were found in the feed, faeces and fresh bedding material. The cubicles were significantly less contaminated than the cubicles at the Research Station. The cows appeared to be clean; the teats were mainly contaminated with sawdust. The results of the RAPD-PCR analyses indicated that the major contamination source of *B. cereus* in milk was the bedding in the deep sawdust bedded cubicles.

On the five additional investigated farms it was found that all had higher spore contents in the milk than that found on the Research Station. All farms using deep sawdust bedded cubicles had high average spore contents in the raw milk (between 63 and 269 spores/l), whereas the farm using sand had the lowest average spore content (33 spores/l milk). High numbers of spores were found in the used bedding material from the deep sawdust bedded cubicles (10⁴ spores/g) and the deep straw-bed (10⁶ spores/g).

The results from all the farms with elevated spore contents in the milk (all farms besides the two housing systems at the Research Station) showed a correlation between the spore contents of the bedding material and the milk ($r = 0.75$; $P < 0.001$), and between the bedding material and the residual rinse water ($r = 0.57$; $P < 0.013$), but not between the residual rinse water and the milk. When the *B. cereus* spores in the bedding exceeded 10⁴ spores/g, there was an increased risk of spore contents above 10² spores/l in milk.

In the feeding experiment when cows were experimentally fed spores, a significant correlation was found between the spore content of the faeces and that present in the milk ($r = 0.78$; $P < 0.001$). The correlation between the spore contents in the milk and the air was 0.61 ($P = 0.001$), and in the faeces and the air 0.67 ($P < 0.001$). There was an elevated risk of an increased spore content in the milk (>10² spores/l) when the spore content of faeces exceeded 10⁴ – 10⁵ spores/g.

***B. cereus* in deep bedded cubicles (Paper II)**

High numbers of *B. cereus* and coliforms were found in the entire bed in deep sawdust bedded cubicles. Lower numbers of *B. cereus* and coliforms were found in the front than in the back of the cubicles ($P < 0.001$). *B. cereus* and coliforms counts at the surface in the back of the bed differed according to the number of days after the bedding had been added to the cubicles ($P < 0.01$); fewer bacteria and spore counts were found immediately after fresh bedding was added than after 2, 3 or 4 days, respectively.

In the laboratory study, the growth of *B. cereus* was affected by the different types of bedding material. Peat and mixtures of peat ($\geq 25\%$) and sawdust with a low pH inhibited the growth of *B. cereus*. When nutrient in the form of urine was added to the different bedding materials, the pH of the peat increased and *B. cereus* growth occurred. Sawdust having a DM content ranging between 20 and 45% did not affect the growth of *B. cereus*. It was subsequently shown that the DM content of sawdust must be higher than 70% to give a water activity below 0.95, which is needed to inhibit the growth of *B. cereus*. *B. cereus* grew less and slower in sawdust with a small amount of faeces compared to larger amounts of faeces.

Adding bedding to the cubicle once daily instead of twice a week resulted in 0.9 log units lower number of *B. cereus* spores in the bedding material before the addition of fresh bedding in the area which was most likely to be in contact with the udders ($P < 0.015$). No difference was found directly after fresh bedding was added. Daily bedding had no effect on the coliform counts in the beds.

After replacing the entire bed in the deep sawdust bedded cubicles, the number of *B. cereus* and coliforms increased very quickly in all parts and at all depths of the beds. Already two weeks after bed replacement, very high bacteria and spores counts could be found.

Teat-cleaning and bacterial spores in milk (Paper III)

In comparison with the control (no cleaning, no forestripping), cleaning teats with a dry paper towel for 10 s reduced the concentration of spores in the milk by 45 to 50%. A 50 to 74% reduction was achieved using different types of moist towels for 10 s. Methods using two towels, soap, or longer cleaning time reduced the bacterial contamination by 85 to 91%. The most effective methods ($P < 0.001$) in reducing milk spore content (96% reduction) were using a moist towel with or without soap followed by drying with a dry paper towel, for a total time of 20 s per cow.

When one of the best cleaning methods was used, the effect of cleaning was independent of the tested contamination carrier (faeces, soil, or sawdust), type of spores (*Cl. tyrobutyricum* and *B. cereus*), or degree of contamination (faeces or extra faeces).

Alley-flooring and hygiene (Papers IV and V)

Electronically-controlled mechanical scrapers placed on top of the slatted flooring in the dairy house improved the floor hygiene by reducing the accumulation of manure in the alleys ($P < 0.001$; Paper **IV**). As a consequence, the faecal contamination in the cubicles was reduced by 39 % ($P = 0.002$). The cleanliness of the udder and teats of cows in the group with scrapers were 27 and 37% cleaner, respectively, compared with the udder and teats of cows in the group without scrapers ($P < 0.001$). The manure contamination within the group without scrapers showed 3.4 times more manure accumulation in the cubicle alley than in the feed alley. The largest amount of manure accumulation in the cubicle alley was found behind the cubicles and in the feed alley close to the wall.

The drainage capacity of concrete slatted floors within the tested range of slat and slot widths depended on the faecal consistency and the floor design (Paper **V**). When loose faeces from high-yielding cows was used, the drainage capacity of the concrete slatted floor depended on the void ratio, with the greater void ratio the better the drainage capacity. When using firmer faeces from dry cows, the drainage capacity depended on both the void ratio and the slot width; shifting to narrower slots required an increase in the void ratio to maintain good drainage capacity.

General discussion

The spore content of raw milk should not exceed 10^2 *B. cereus* spores/l in order to guarantee the shelf-life of pasteurized milk under Swedish conditions. The first investigation at the Alnarp Research Station as presented in Paper I showed that it is possible to produce raw milk with a small number of *B. cereus* spores ($< 10^2$ spores/l) during the housing period, both in loose housing systems or tie-stalls. Neither feed, air, faeces, bedding, nor milking equipment contributed to elevated milk spore contents. Most farmers manage to deliver high quality milk during the period when cows are housed inside; however, problems can occur.

Sources and routes of contamination

In the investigations on six commercial dairy farms with different types of housing systems in Paper I high concentrations of *B. cereus* spores in the milk occurred on some of the farms during the housing period. Samples from these farms, analyses with RAPD-PCR on one of the farms, and a feeding experiment gave some valuable information about possible contamination routes (Paper I). The results are discussed below together with results published in previous investigations.

Feed and faeces

In Paper I, the spore content of *B. cereus* both in the feed and faeces was found to be low, as often found by others (Slaghuis *et al.*, 1997; Christiansson *et al.*, 1999; Vaerewijck *et al.*, 2001). However, some instances of elevated spore contents in the feed have previously been found. Spent grain from the brewery has been shown to contain more than 10^6 spores/g (Barkley & Delaney, 1980; Torp *et al.*, 2001). It was noted that *B. cereus* can grow during the later stage of the aerobic spoilage of silage (Lindgren *et al.*, 1985), and silage was considered by some researchers to be a contamination source of aerobic and *B. cereus* spores (Torp *et al.*, 2001; te Giffel *et al.*, 2002). The highest reported levels of *B. cereus* spores in silage were 10^4 spores/g (Crielly *et al.*, 1994; te Giffel *et al.*, 1995; Torp *et al.*, 2001).

In the feeding experiment (Paper I) when the cows were fed *B. cereus* spores, there was a positive correlation found between the *B. cereus* spore content of the feed and the faeces, as previously found by Labots *et al.*, (1965). In Paper I it was observed that a considerable portion of the spores was unaffected by passing through the digestive tract. Faeces containing more than 10^4 - 10^5 spores/g resulted in a degraded milk quality with spore concentrations above 10^2 spores/l milk. More than 10^4 spores/g faeces was found in groups of cows fed a large quantity of spores, corresponding to 30 kg silage with a spore content of 3×10^5 spores/g. Visser *et al.*, (2007) made a simulation model where silage was the main contamination source during the housing period. They noted that high spore concentrations in milk could occur when the initial spore concentration in silage was $> 10^3$ spores/g and when the pH of the feed ration was above 4.9 (*B. cereus* grow at a pH above 4.9). In addition, the extent of milk contamination with

bacterial spores also depended on the amount of faeces on the teats at milking. It could be concluded that giving highly spore contaminated feed results in highly spore contaminated faeces hence a major source of spore contamination of the raw milk.

Bedding materials

The presence of large numbers of aerobic spores (McKinnon & Pettipher, 1983; Slaghuis *et al.*, 1991), and of *B. cereus* spores (Crielly *et al.*, 1994) have previously been reported in used bedding materials. In Paper I high numbers of spores were found in the bedding on some farms. A relationship between the number of spores in the bedding and in the milk was observed. When the concentration of spores in the bedding exceeded 10^4 spores/g, there was an increased risk of having too high a spore content in the milk ($> 10^2$ spores/l).

Large numbers of *B. cereus* spores were found in deep sawdust bedded cubicles (Papers I and II). The spore counts could be as high as those found in the soil during the grazing period (Christiansson *et al.*, 1999). It was noted that the bedding material remained in the cubicles for a long period of time, and that there was an active growth and sporulation of *B. cereus* in these beds (Papers I and II). The RAPD-PCR analyses in Paper I showed that the bedding material was the major source of spore contamination in the milk. Very high numbers of spores were also found in some samples obtained from a deep straw-bed (Paper I). In tie-stalls and in cubicles with rubber mats and bedded with a small amount of sawdust which only remained for a short period on the stall surface, bacterial growth to large numbers did not occur (Paper I). When the lying area consisted of a bedding material which remained for a long time in the cubicles or beds, the bedding could be a major source of *B. cereus* milk contamination. These types of deep beds are mainly used in various kinds of loose housing systems and this circumstance could possibly explain why some people had associated elevated spore contents of *B. cereus* in milk during the housing period to loose housing systems.

Inside air

The air in the animal barn was suggested to be a source of contamination by Stewart (1975). Low number of spores was found in the inside air in Paper I. Less than 0.5 spores/l air was found in the investigated animal barns, even when high numbers of spores were found in the bedding material. During the feeding experiment at the most 1.7 spores/l was found in the air. However, at the same time, the spore content of the faeces ($>10^5$ /g) and the milk (between 10^2 - 10^3 spore/l) was high. Assuming that 10 litre of air/l milk would be sucked into the milking equipment (Christiansson *et al.*, 1999), the spore contribution of the air to the total contamination of the milk would be low in comparison to that of the faeces. This is in agreement with more recent studies by te Giffel (1995) and Christiansson *et al.* (1999). The results of the RAPD-PCR analyses in Paper I confirmed that the air did not appear to be an important source of spore contamination in the milk.

Milking equipment

During the feeding experiment in Paper I, the milking equipment was observed to contribute with a low number of spores to the milk. On the farm with high numbers of spores in the bedding and in the milk, high numbers of spores could also be found in the rinse water (farm 2, Paper I). It was not possible to determine whether the bedding was the only source or whether the milk also was contaminated by the milking equipment. Using RAPD-PCR analyses, it was concluded that the bedding material was the main source of the milk contamination. However, the rinse water collected at two different places did not show the same results, and this could possibly reflect that the equipment had become contaminated. An older investigation (Labot *et al.*, 1965) showed that the milking equipment was a source of contamination. Using the RAPD technique, Christiansson *et al.* (1999) found a low degree of *B. cereus* spore contamination in the milking equipment in connection with a cleaning problem. When the cleaning procedure is inadequate, the milking equipment could contribute to spores in the milk.

Deep bedded cubicles

In Paper II both *B. cereus* and coliforms were present in high numbers in the entire deep sawdust bedded cubicles. On average, from 10^4 up to 10^6 spores/g were found in individual samples from the upper layers at the back part of the beds, the area which could be in contact with the cows' udders. According to Paper I, this presented a risk for having high milk spore contents ($>10^2$ spores/l).

Type of bedding material

The laboratory experiments in Paper II showed that the growth of *B. cereus* differs in the different types of bedding materials. An extensive growth of *B. cereus* occurred in straw, however, the results in sand and sawdust varied. Some time had elapsed between the two experiments carried out, and sand and sawdust from different sources may have been used, possibly affecting the results. A difference in bacterial growth in different sources of the same type of bedding has been found previously (Zhener *et al.*, 1986; Bernhard *et al.* 2003). High aerobic spore counts in straw and in sawdust have been observed by Slaghuis *et al.*, (1991); the numbers found were higher in straw than in sawdust. In the laboratory experiment reported in Paper II, the growth of *B. cereus* was inhibited in peat, probably due to the low pH. *B. cereus* grows in a pH range between 4.9 and 9.6 (Kramer & Gilbert, 1989). When urine was added to the peat, the pH increased and bacterial growth occurred. It is not known if the inhibitory effect of pH in peat will prevail over time under barn conditions, and this should be further investigated. It had been observed that adding lime, alkaline or acidic conditioners to sawdust or recycled manure as bedding affected the pH, but had only a short term limiting effect on the bacterial counts in the bedding (Hogan & Smith, 1997; Hogan *et al.*, 1999, 2007).

The use of inorganic bedding such as sand has been shown to result in lower counts of environmental mastitis pathogens in the bedding, as compared to those found when using organic bedding, such as sawdust (Fairchild *et al.*, 1982; Hogan *et al.*, 1989). Alternative bedding material such as sand and peat could thus provide less favourable conditions for the proliferation of *B. cereus*. However, Bernhard *et al.*, (2003) found 10^4 *B. cereus*/g in recycled sand used for bedding. Different bedding materials might also have different properties of adherence to the teats and thus affect the possibility of contaminating the milk via that route differently (Zdanowicz *et al.*, 2004).

Effect of bedding conditions on growth of B. cereus

The water activity should be less than 0.95 to inhibit the growth of *B. cereus* (Kramer & Gilbert, 1989). In order to achieve this, the DM content of sawdust must be higher than 70%, according to the curves of adsorption and desorption shown in Paper II. Zdanowicz *et al.*, (2004) found that the DM content correlated to the bacterial counts in sawdust bedding. However, in their experiments kiln-dried sawdust was used and the lowest DM found was 71.7%. The DM content in fresh sawdust varies, dependent on the source and storage. Under the conditions in Papers I and II when sawdust had a DM of 45 -53% prior to use, which is considerably below 70%, it was not possible to maintain the DM in the beds above 70%.

The laboratory experiments in Paper II also indicated that the availability of nutrients in the form of faeces in the beds enhanced the growth of *B. cereus*. This was also found by Zdanowicz *et al.*, (2004). These results showed the importance of keeping the cubicles clean and dry.

Management to minimize bacterial growth

The deep sawdust bedded cubicles were shown to contain large numbers of spores, which varied between a maximum value observed before giving fresh bedding and a minimum value found directly after the fresh bedding had been added to the cubicles (Paper II). The sawdust beds on the investigated farm were well managed (Paper II); fresh bedding material was added twice a week with a large amount of sawdust given; the beds were cleansed thrice daily, and all the sawdust in the beds was replaced once a year. It was not possible to permanently decrease the amount of spores in the beds through changes in the management. Daily bedding as compared to bedding twice weekly had a small effect, and resulted in 0.9 log units lower number of spores in the upper layers of the beds before the fresh bedding was added (Paper II). For both treatments were lower numbers of spores found directly after fresh bedding was added than one and three/four days after bedding was added, respectively. The more frequent bedding is added to the beds the upper layers of bedding will have a decreased number of spores during a greater part of the time. The fresh bedding should be placed at the back of the cubicles where the cow's udder comes in contact with the bedding, and mixing the fresh bedding with the old should be avoided. Probably the effect

varied with the amount of bedding given: the more bedding added, the better the effect.

Daily bedding had even less influence on the coliform count than on that of *B. cereus*. The counts were high already after one day, as also observed by Hogan & Smith (1997).

As shown in Paper II, replacing the entire bed only temporarily reduced the spore contents of the beds. Already after two weeks high numbers of spores were found in the beds. In this experiment, the beds were replaced in a limited number of the cubicles in a section with 140 cubicles. *B. cereus* already occurred in high number in the surrounding beds and was easily introduced to the newly replaced beds. An entire bed replacement for all the cubicles and a thorough cleaning of the section might have improved the lasting time for reduced spore content. Apparently an increase in the spore content of the beds occurred with time even if entire bed replacement was made once a year. This phenomenon is illustrated by the higher numbers of spores found in the beds in Paper II, than in the study in Paper I, conducted on the same farm two years previously.

It should be noted that much depends upon the quality of the fresh bedding material. This could vary and also the storage conditions could affect the bacterial contents (Zhener et al., 1986). In paper II, in the study of entire bed replacement very high numbers of coliforms were found in fresh sawdust prior to use as bedding, and consequently the coliform counts were high in the beds already from the start. However, the content of *B. cereus* spores in fresh sawdust was low ($<10^2$ spores/g) in all studies (Papers I and II).

Teat-cleaning

It is evident that the major route of *B. cereus* spore contamination of milk is via the teats. Spore contaminated bedding material and faeces from the beds and soil when cows are on pasture will adhere to the udder and teats (Christiansson *et al.*, 1999; Paper I; Paper II). In addition, other bacteria and *Clostridium* spores are transmitted to the milk via the contaminated teats (Hogan et al., 1988, Stadhouders and Jørgensen, 1990). The presence of mastitis pathogens on teat ends at milking has been correlated to incidences of intramammary infections (Pankey, 1989). Therefore, the udder and teats of the cows have to be cleaned before milking.

Method for evaluation of teat cleaning methods

Worldwide, different premilking cleaning methods are used and their efficiency varies. The method used in Paper III to experimentally contaminate the teats with *Cl. tyrobutyricum* spores was a practical way of evaluating the effectiveness of different premilking teat-cleaning methods. Anaerobic spores are excellent microbiological cleaning indicators because they do not grow aerobically and are not killed by the cleaning agents. Furthermore, this indicator was actually the object we wanted to study. Other tracer substances, such as cobalt and poppy

seeds, have been used in studies evaluating teat-cleaning in automatic milking (Knappstein *et al.*, 2002, Knappstein *et al.*, 2004). The use of cobolt was not successful, but the use of poppy seed showed difference in cleaning efficiency between different brands of automatic milking systems. In manual teat-cleaning method similar to those used in paper **III**, they found larger reduction of poppy seed than we found of spores. The adhesion of poppy seed to the teats is probably less strong than spores.

The method used in Paper **III** uniformly contaminating the teats, making it possible to reduce the number of cows and samples in the experiments, and providing more power of resolution between cleaning methods. It was important to use contamination mixtures containing large concentrations of spores and to minimize influence of natural contamination from bedding and faeces.

Teat cleaning methods

The results presented in Paper **III** were clear and easy to interpret. Differences were shown in cleaning efficiency between the present commonly used teat-cleaning methods, papers, clothes, and soap and foam used in Sweden. The results agreed with those of previous studies, which mainly focussed on the reduction of bacterial contamination (Galton *et al.*, 1982, 1986; Adkinson *et al.*, 1991; Ingwa *et al.*, 1992), and also with those studies which had dealt with spore contamination (Stadhouders & Jørgensen, 1990; Rasmussen *et al.*, 1991). In general the cleaning results depended on: the use of dry or moist cleaning, towel quality, use of soap, and cleaning time.

Type of towel

Cleaning teats with a wet or moist towel was often better than cleaning with a dry towel (Galton *et al.*, 1986; Paper **III**). Thin wet paper towels were easily ripped apart and were less efficient (Rasmussen *et al.* 1991, Paper **III**). In Paper **III**, wet mechanically stronger paper towels were as good as wet washable towels made of synthetic material or cotton.

Soap

Washing the teats with a hose should always be followed by careful drying (Galton *et al.*, 1984). The use of different sanitizers or disinfectants improved the cleaning results as compared with using only water from a hose and dry cleaning (Adkinson *et al.*, 1991; Ingwa *et al.*, 1992). When the teats were sprayed or dipped with soap followed by drying with a paper towel, a better reduction in spore contamination was obtained than just by using a dry or wet towel, but not better than using a wet towel followed by drying with a paper towel (Galton *et al.*, 1986; Paper **III**). Using foam gave better results than spray (Paper **III**), possibly due to the application method or to the differences in the composition of compounds in the soap solutions. Best results were obtained when soap was applied by a moist towel followed by drying with a paper towel (Paper **III**). This method produced similar results with or without soap. With longer manual cleaning time, using soap appeared to be of less importance. Using a disinfectant entailed a risk of having

other residuals in the milk (Galton *et al.*, 1984; Rasmussen *et al.*, 1991). In Sweden, soap solutions are allowed but disinfectants are not allowed to be used on the teats before milking (Svensk Mjlk kemikalierd, 2007).

Cleaning time

A longer cleaning time of 20 s had a better effect than a cleaning time of 6 or 10 s (Rasmussen *et al.*, 1991, Paper III). Using one towel for 20 s on severely contaminated teats, however, was not as good as using two moist towels for 10 s each (Paper III). With one towel, the contamination was likely to only be spread about, not removed upon prolonged cleaning time.

Proper premilking teat-cleaning

The best method found in Paper III was using a moist towel with or without soap followed by drying with a dry paper towel for 20 s (96% reduction). Problems with *B. cereus* spores contaminating the milk due to soil contaminated teats during the pasture season, or due to bedding contamination during the housing period could be reduced by using good premilking cleaning routines. It was shown in Paper III that the cleaning effect was similar for both *B. cereus* and *Cl. tyrobutyricum* spores and the efficiency was not affected by the type of contamination (faeces, soil, or sawdust). The degree of faecal contamination on the teats did not affect the cleaning results. The more faecal and spore contamination of teats there was, the more intense the teat cleaning was needed to produce milk having a low spore concentration. However, no cleaning method removed the spores 100%.

A longer premilking teat-cleaning is considered by some farmers to be too time consuming. However, proper milking routines include prestimulation of the udder to trigger the milk ejection reflex. The optimal duration of prestimulation in order to receive an immediate and continuous milk flow has been shown to be 20 to 90 s, depending on how full the udder is at the time (Weiss & Bruckmaier, 2005). Longer prestimulation times for udders that were not full also led to a reduced total vacuum load on the teats when milking. Longer prestimulation time could also increase the milk yield. Rasmussen *et al.* (1992) reported increased milk yield for Danish Jersey cows, but not for American or Danish Holstein cows in response to longer prestimulation time. An optimal prestimulation of 20 to 30 s was suggested. Sandrucci *et al.* (2007) found in a field study that proper udder preparation (forestripping, teat-cleaning, and predipping) resulted in a greater milk yield per milking, greater peak milk flow rate, shorter total milking time and lesser bimodality, than did poor preparation. Presumably, the total milking time will not be prolonged when using a cleaning time of 20 s plus forestripping.

By using a model simulation, Vissers *et al.* (2006) showed that the most effective milking strategy to reduce the milk spore content should be only to use time consuming cleaning method on visibly dirty cows and less time consuming methods on cows with no visible dirt on the teats. However, care must be taken when the bedding is the contamination source because the teats can appear to be

clean but still be contaminated with a high number of bacteria and spores (Paper I). On the other hand, Rasmussen *et al.* (1992) showed that there was an increase in milk yield when a standardized milking routine was used on all the cows as compared with a variable milking routine.

Factors affecting cow cleanliness

Cows and especially the udder region should be maintained in conditions that avoid unnecessary soiling for many reasons. Clean cows save time and effort when milking, since the cleaning prior to milking can be less extensive. When the cows' teats are highly contaminated with spores, there is still a risk of high spore contamination in the milk, despite a thorough teat cleaning (Paper III). A relationship between the cleanliness of the cows and the rate of subclinical mastitis has been reported (Schreiner & Ruegg, 2003; Renau *et al.*, 2005), and cleaner cows and cleaner housing were found to be correlated to a lower SCC in the milk (Barekema *et al.*, 1998; Köster *et al.*, 2006). Consequently it is of importance to keep the teats and udder clean, not only to produce high quality milk, but also to keep the animal healthy. Furthermore, consumers are taking more interest in the source of production and the welfare of the animals involved. Udder and teats should be clean 24 hours a day.

Cubicles

A clean lying area is essential for the cleanliness of the udder and teats of the cow. Proper size and design of the cubicles lessen the amount of faeces present in the lying area due to direct defecation from the cows when lying or standing in the cubicles (Tucker *et al.*, 2004; Tucker *et al.*, 2005). Proper management of the lying area is of importance to prevent bacterial growth and sporulation in the beds, as discussed before. Frequent bedding and cleaning intervals of the beds are needed to prevent the faeces and urine from supplying nourishment for bacterial growth. The more frequently the bedding is added to the cubicles, the more often the udder and teats can come in contact with clean bedding instead of soiled bedding. There must also be sufficient bedding in the lying area to embed milk leakage and the faeces brought into the cubicles via the claws of the cows. In Paper IV it was shown that a lower accumulation of manure in the alleys by mechanically scrapers reduced the amount of contamination with faeces brought into the cubicles. This difference in faecal contamination in the cubicles has a significant effect on the cleanliness of the udder and teats.

Alleys

Slatted floors are commonly used in Swedish cubicle houses and the floor is cleansed by self-cleaning via the feet of the cows trampling down the manure. The cleanliness of the slatted floor depends on the floor design, faecal consistencies, and number of steps taken by the cows (Boxberger & Pfadler, 1980; Boxberger & Pfadler, 1982; Paper V). The alley area per cow has to be limited because the cows have to trample down the manure, which they would not be able to do if the area was large. In Paper IV a large accumulation of manure was found to be present

along the walls and behind the cubicles; this has to be removed manually to prevent cubicle contamination. Installing a mechanical scraper on top of the slatted flooring solved these problems, and reduced the manure accumulation on the alley floor (Paper **IV**). The use of scrapers on slatted floors results in the hygiene being independent of cow traffic and allows larger floor areas per cow to be used.

The cleanliness of the slatted floors where there are no scrapers depends on the void ratio of the floor (Paper **V**). For lactating dairy cows with loose faeces, the greater the void ratio the better the drainage capacity and the cleaner the floor. For heifers and dry cows with firmer faeces shifting to narrower slots and smaller slats require increased void ratio to maintain good drainage capacity. However, the design of the slatted floor should also consider good support for the claws and pressure distribution of the foot-floor contact (Nilsson *et al.*, 2006). Openings must not be too wide. In the further development of slatted floors with smaller slats and slots to better meet the requirements of the pressure distribution under the claws, it is of importance not to reduce the drainage capacity and thus lead to a deterioration in the cleanliness. If scrapers are used on top of the drainage floor; the floor can be designed in a more animal friendly way.

Conclusions

Large numbers of *B. cereus* spores can occur in the raw milk during the housing period of the cows (Paper I):

- Bedding material that remains for a long time in cow beds can contain large numbers of *B. cereus* spores and contaminate the milk via contaminated teats.
- Feed with a high *B. cereus* spore content can be a source of contamination in milk via the faeces and contaminated teats.
- Contaminated milking equipment can contribute to the presence of *B. cereus* spores in the milk.
- The air around the cows does not appear to have any major influence on the *B. cereus* spore content in the milk.

In deep sawdust bedded cubicles, a considerable growth of *B. cereus* can occur and high amounts of spores can be found. The type of bedding material, pH, and the availability of nutrients in the form of faeces affect the growth of *B. cereus* in the beds. The possibility of permanently reducing the spore levels in the deep sawdust beds through management is limited. Management can to some extent reduce the spore content by frequent addition of fresh bedding material and cleaning (Paper II).

The cleanliness of the cows' udder and teats plays an essential part in the contamination routes and thus maintenance of hygiene in their housing is of the greatest importance. The results of the present series of studies show not only that there are differences in cleaning efficiency between different premilking teat-cleaning methods, but also that it is possible to control the cleanliness of udder and teats by good hygienic conditions in alleys and cubicles.

The method to experimentally contaminate teats with *Cl. tyrobutyricum* spores was a practical and useful way to evaluate the effectiveness of different teat-cleaning methods (Paper III)

When teats are spore contaminated (*B. cereus* or *Cl. tyrobutyricum*) different teat-cleaning methods affect the amount of spores in the raw milk (Paper III):

- Using a moist towel is better than cleaning with a dry towel.
- Using a spray or dip with soap before drying with a dry paper towel improves the cleaning results.
- Longer manual cleaning time improves the cleaning results.
- Using two moist towels is better than using one moist towel.

- The best method is using a moist towel followed by drying with a dry paper towel. In herds with spore problems this teat-cleaning routine should be applied.
- The effect of the teat-cleaning method is not influenced by the nature of the contamination (soil, sawdust, or faeces), type of spores, or degree of contamination on the teats.
- Not even the best cleaning method tested in this study removes all the bacteria and spores.

Scrapers on slatted floors improve the hygiene of the alley floor (Paper IV). Decreasing the manure accumulation on the alley floors reduces the risk of faecal contamination in the cubicles, and improves the cleanliness of the udder and teats.

The design of the slatted floor affects the drainage capacity (Paper V). For lactating dairy cows with loose faeces, the greater void ratio of the slatted floor the better drainage capacity and the cleaner the floor, irrespective of the slat and slot width. However, the floor design also has to consider good support for the claw, and the pressure distribution between the claw and the slatted floor. To maintain good drainage capacity on slatted floors for heifers and dry cows with firmer faeces an increase in void ratio is required when shifting to narrower slots. If scrapers are used on top of the drainage floor; the floor can be designed in a more animal friendly way.

Practical recommendations

To reduce the risk of *B. cereus* contamination in raw milk during the housing period the following recommendations are given:

- Feed with silage of high microbiological quality.
- Add bedding frequently to lying areas using sufficient bedding material to embed milk leakage and faecal contamination. The lying area should also be cleaned frequently to keep the area clean and dry.
- Keep alley floors clean by frequent scraping.
- When deep bedded cubicles or deep straw-beds are used, special consideration should be taken to frequent bedding and cleaning of the beds, and to careful premilking teat-cleaning.
- The udder and teats should be cleaned before milking. In herds with spore problems with the feed or bedding, premilking teat-cleaning using a moist towel with or without soap followed by drying with a dry paper towel is recommended.
- Clean the milking equipment properly.

Areas for future research

Large amounts of *B. cereus* spores in deep sawdust beds were found, but they were also noted in some samples from a deep straw-bed. The laboratory experiments indicated that *B. cereus* did not grow as well in alternative beddings such as peat and sand. Further investigations are needed into the presence of *B. cereus* in different bedding materials, such as sand, straw and peat. It is also of interest to determine to what extent the different bedding materials and spores adhere to the udder and teats.

The result of these studies showed that *B. cereus* contaminated feed could be a significant source of the spore contamination in raw milk, when the number of spores excreted in the faeces exceeded 10^4 - 10^5 spores/g. Visser *et al.* (2007) developed simulation models and considered silage with $>10^3$ spores/g to be a risk to the milk quality. Further investigations are needed to determine the occurrence of *B. cereus* in silage, and the concentration of spores in silage that could be considered as a risk to the milk quality.

When the udder and teats of the cows are contaminated, the last possibility of preventing milk contamination is at the milking. To improve teat-cleaning and decrease the work load of the milker, the development of effective automatic premilking teat-cleaning devices in the milking parlour should be encouraged and evaluated using standardized teat contamination methods.

More frequent bedding improves the hygiene in the cubicles. Development of an automatic distribution system where the bedding could be delivered at any time would simplify the bedding routines and increase the bedding frequency.

The cleanliness of the slatted floors was improved with the installation of mechanical scrapers. When scrapers are used, the drainage capacity of the floor is not as important as it is without scrapers. It would be interesting to develop a new alley floor system with a draining floor that has a claw friendly design and scrapers on top, which would improve both the hygiene and the welfare of the cows.

Populärvetenskaplig sammanfattning

Den sporbildande bakterien *Bacillus cereus* kan förekomma i mjölk och sporererna överlever pastörisering. *B. cereus* är den viktigaste hållbarhetsbegränsande bakterien i konsumtionsmjölk. Bakterien är vanligt förekommande i jord och hållbarhetsproblemen med mjölk är som störst under betessäsongen då kornas spenar kan förorenas med jord. Periodvis kan dock problem även förekomma under stallperioden.

Målet med denna avhandling var att identifiera föroreningskällor och spridningsvägar av *B. cereus* i kornas närmiljö under stallperioden samt att undersöka om man genom olika åtgärder kan minska sporförekomsten i mjölken.

I en inledande serie av studier undersöktes förekomsten av *B. cereus* i prov från foder, gödsel, vatten, rent strömmaterial, strömmaterial från bås och bäddar, luft, skölvatten från mjölkkningsanläggning och från tankmjölk. I större eller mindre omfattning togs prov på 7 olika gårdar med olika typer av inhysningssystem. Endast låga halter av sporer hittades i foder, gödsel och luft, och de ansågs inte vara av betydelse som föroreningskällor på dessa gårdar.

På några av gårdarna fanns höga halter av sporer i strömmaterial från kornas liggytor. På en utav dessa gårdar fanns också höga halter av sporer i skölvatten från mjölkkningsanläggningen. Det fanns ett samband mellan sporhalten i strömmaterial på liggytor och sporhalten i mjölken. Med hjälp av RAPD-PCR analyser som ger ett genetiskt fingeravtryck av bakteriestammarna kunde vi dra slutsatsen att det var strömaterialet i liggbåsen som var huvudkällan för sporförekomsten i mjölken. Höga halter av sporer hittades i djupa sågspånsbäddar i liggbås och i en del prov från en djupströbbädd med halm.

I ett separat försök där kor avsiktligt utfodrades med sporer visades att om korna konsumerade höga halter av sporer fanns det höga halter av sporer i gödseln. Genom gödsel nedsmutsning av spenar ökade sporhalten i mjölken. Följaktligen kan foder med höga sporhalter vara en viktig föroreningskälla.

För att genom olika skötselrutiner försöka minska förekomsten av sporer i de djupa sågspånsbäddarna undersöktes dessa ytterligare i nya studier. Lika höga halter av sporer som man tidigare har funnit i jord fanns i de djupa sågspånsbäddarna. När strömaterialet ligger kvar i båsen eller i bäddar under en längre tid finns det tillfälle för *B. cereus* att tillväxa och sporulera.

I laboratorieundersökningar visades att typ av strömmaterial påverkade tillväxten av *B. cereus*. Torv och torv/spån-blandningar hämmade tillväxten av *B. cereus*, troligen på grund av sitt låga pH. Om denna effekt kvarstår under längre tid i stallmiljö behöver undersökas ytterligare. Mängden gödsel i strömaterialet påverkade tillväxten och *B. cereus* växte långsammare och mindre i sågspån med låga gödselhalten jämfört med i sågspån med höga gödselhalter.

I ett försök där ströning skedde dagligen istället för 2 ggr per vecka minskade sporhalten något i den översta delen av bäddarna och ju oftare man strör ju större del av tiden kommer juvret i kontakt med nytt renare spån. I ett annat försök då hela bädden grävdes ut och ersattes med nytt spån minskade sporhalten bara tillfälligt. Redan efter två veckor fanns höga sporhalter i bäddarna igen.

Strömmaterial och gödsel förorenar mjölken genom nedsmutsning av spenar. Det är därför viktigt att spenarna är rena vid mjölkningen. I försök där spenarna förorenades med en gödselblandning innehållande clostridiesporer undersöktes rengöringseffekten av olika avtorkningsrutiner före mjölkning. De visade att den effektivaste avtorkningsmetoden var att först torka med en fuktig trasa i 10 s och sedan torka av med en torr papersduk i 10 s. Då reducerades sporhalten med 96% jämfört med om ingen avtorkning alls hade skett. I ytterligare försök visades att metoden var lika effektiv oavsett om spenarna var förorenade med *B. cereus* sporer eller clostridiesporer och om spenarna var förorenade med sågspån, jord eller gödsel. Denna avtorkningsmetod bör användas i besättningar med sporproblem.

Trots en bra avtorkningsrutin blir man inte av med alla sporer till 100%. Därför är det viktigt att kornas juver och spenar hålls så rena som möjligt mellan mjölkningarna. I ett försök med mekaniska skrapor på spaltgolv där skraporna gick 8-12 ggr/dygn minskades mängden gödsel som ligger kvar på spaltgolvet och mängden gödsel som korna drog in med klövarna i liggbåsen minskade med 39%. Kornas juver blev 27%, och spenarna 37% renare än när skrapor inte användes.

Spaltgolvets dräneringsförmåga undersöktes i ett laborationsförsök. Dräneringsförmågan påverkas av gödselkonsistensen och när lös gödsel från högproducerande mjölkkor användes var dräneringsförmågan beroende av andelen öppningar i golvet. Ju högre öppningsprocent, ju bättre dräneringsförmåga, oavsett stav och spalt bredd. När fastare gödsel från sinkor och kvigor användes var dräneringsförmågan beroende både av öppningsprocent och spaltbredd, minskar man spaltöppningen måste öppningsprocenten ökas för att bibehålla samma dräneringsförmåga.

Resultaten visade att mjölken kan förorenas med *B. cereus* sporer genom att spenarna är nedsmutsade med gödsel och strö som innehåller höga halter av sporer. Det är av stor betydelse att hålla kornas juver och spenar rena inte bara med effektiva avtorkningsrutiner utan också genom att ha en god hygien på liggytor och i gångar.

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